A comparative study of the potential for human retinal pigment epithelium transdifferentiation utilising an embryonic chick model of the phenomenon.

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PhD Thesis

I, Matthew John Kevin Smart, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Abstract

The retinal pigment epithelial (RPE) cells of several species, including: chicken, rat, mouse and newt, have been observed to undergo a phenomenon known as *transdifferentiation*. This involves the re-specification of the RPE phenotype towards a neuroretinal, or lens phenotype, in response to various cues, including basic fibroblast growth factor (bFGF). Transdifferentiation has yet to be described in human RPE cells, however, this investigation has demonstrated that human cells, including primary fetal, and human embryonic stem cell-derived (HESC), RPE, may retain the capacity to undergo some level of bFGF-mediated transdifferentiation. It appears that the process is likely comparable to that observed in the embryonic chick model of the phenomenon, given that human transdifferentiation appears to be restricted to the earliest stages of RPE development (approximately 6 weeks). Additionally, the features of transdifferentiated RPE observed in the earliest available human tissue are shown to resemble that of a similar stage in chick development (HH27), which is shown to display limited transdifferentiation for the first time, in contrast with previous studies which report the loss of potential for transdifferentiation at this stage. It remains unclear as to why RPE cells lose the capacity for transdifferentiation with development, however, it appears to be linked to, but not exclusively a result of, a loss in the expression of Pax6 across the RPE monolayer, given both capacity for transdifferentiation, and Pax6 expression, are both variable in different regions of the same monolayer, in the chick model of transdifferentiation. Known RPE augmentation signaling pathways, including bone morphogenic protein (BMP) and Sonic hedgehog (Shh) were analysed for their potential involvement in the restriction of transdifferentiation, however, neither appeared to be directly involved. Further studies in the embryonic chick model of the phenomenon will be necessary to unlock this potential in human RPE cells.

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First of all I'd really like to thank Pete and Ant for giving me the opportunity to join the lab and do a PhD with them in the first place. I couldn't have picked a better lab to work in and I've really enjoyed the atmosphere, the science, and the people I've been surrounded with and I genuinely think it's helped me become a better bloke. I'd like to thank them both for being top guys to hang around with, as well as great scientists who've taught me plenty over the years - not to mention led me on more than my fair share of merry adventures! I have to sincerely thank them/apologise for putting up with the constant revisions of my thesis, including helping me put it together in the first place. You guys have always encouraged me to question things and follow my own nose when trying to figure things out and I'll never forget the mantra you both left me with, namely, try keep it as simple and straight forward as you can! I feel like this will stand me in good stead going forward and hopefully make me a better scientist down the line. I'd especially like to thank Pete for backing me in the lab with coin, post-grant etc. It's much appreciated and hopefully I can go some way to paying it back in the near future! Put me down for another book chapter haha!

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Abbreviations

AMD – Age-related macular degeneration

AP2α – Activating enhancer binding protein 2 alpha

bFGF – Basic fibroblast growth factor

BMP – Bone morphogenic protein

Chx10 – Ceh-10 homeodomain containing homolog

CMZ – Ciliary marginal zone

CRX – Cone-rod homeobox protein

CS – Carnegie stage

cVax – Ventral anterior homeobox 2

DCT – Dopachrome tautomerase

ERK – Extracellular signal-related kinase

F – Fetal stage

FGF – Fibroblast growth factor

FGF-R1 – Fibroblast growth factor receptor 1

HH – Hamburger-Hamilton stage

HESC – Human embryonic stem cell

HESC-RPE – Human embryonic stem cell-derived RPE

HuD – ELAV-like protein 4

IPE – Iris pigment epithelium

iPS – Induced pluripotent stem cell

IRBP – Inter-retinoid binding protein

MERTK – C-mer proto-oncogene tyrosine kinase

MitF – micropthalmia-associated transcription factor

MMP115 – Matrix melanosomal protein 115

NRL – Neural retina leucine zipper

Optx2 – Optic six gene 2

Otx2 – Orthodenticle-homeobox protein 2

Pax6 – Paired box 6

Pmel17 – Melanocyte precursor protein 17

RPE – Retinal pigment epithelium

Shh – Sonic Hedgehog

Sox2 – Sex determining region homeobox 2

Tbx2 – T-box transcription factor 2

TGFβ – Transforming growth factor beta

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Chapter 1 - Introduction

1.1 Thesis outline:

This thesis will outline studies concerning the capacity for transdifferentiation of RPE cells towards a neuroretinal phenotype in both an established, embryonic chicken model of the phenomenon, as well as several different human RPE sources. Chapter 1 will outline the need for sources of human retinal cells, in addition to the known developmental processes which have been implicated in the regulation of transdifferentiation in RPE cells. Chapter 2 will outline the general materials and methodology used to analyse the development and transdifferentiation of RPE cells. Chapter 3 describes the expression of different retinal markers in both developing native chick retina, in addition to transdifferentiated chick RPE explants. Chapter 4 attempts to identify candidates that are responsible for the inhibition of the capacity for transdifferentiation, in the hope that this would lead to enhanced culture conditions which would promote the transdifferentiation of RPE cells which appear to have lost the capacity for transdifferentiation under standard conditions. Chapter 5 outlines a comparative study of the potential for transdifferentiation in primary human fetal RPE cells, whereas chapter 6 analyses the capacity for transdifferentiation in human embryonic stem cell derived RPE cells using the established culture system. Chapter 7 discusses the overall outcomes of the whole investigation, including what line of questioning would be best to follow in future studies.

1.2 Eye development:

The eye is a very complex organ that is responsible for sensing light. Light enters the eye globe through a window (cornea) at the front of the eye and is focused onto the back of the eye by an adjustable lens which can change shape in order to focus on objects at different distances. At the back of the eye, several different layers of cells are present, which perform

specific roles that are crucial for vision. The most simple of these layers is a pigmented monolayer known as the retinal pigment epithelium (RPE), which lies beneath, and in contact with, a thicker, multi-layered, cellular structure known as the neural retina. The neural retina contains seven different cells types which each have a specific role in vision, and these cells are specifically organized into cellular layers which are dependent on their function. Neural retinal cells comprise the retinal circuitry responsible for sensing photons of light entering the eye, and transferring this visual information to the brain. It does this via photosensitive molecules in the photoreceptor cells, known as opsins, which produce an electrical signal when exposed to specific wavelengths of light. Crucially, the photoreceptor cells are localized to the outer nuclear layer (ONL) of the neural retina, which is immediately adjacent to, and in contact with, the RPE monolayer. This contact is crucial for the proper function and maintenance of the photoreceptor cells, which require a functional RPE monolayer in order to survive.

The eye is one of the first organs to develop during embryogenesis, and its early development is largely conserved across a number of different species (for review see (Chow and Lang, 2001, Fuhrmann, 2010)and an overview of known chick eye development is summarized in Fig. 1.2). The eyes develop through optic primordia, which bud off from the diencephalon (Fig. 1.1A) and extend towards the over-lying surface ectoderm. These globes are known as the optic vesicles, which consist of a single layer of multi-potent, progenitor cells that will eventually specify both the RPE and neural retina (Fig. 1.1B). Once the optic vesicles have formed and are in contact with the over-lying surface ectoderm, the vesicles begin to invaginate to produce the optic cup structures. Invagination results in a bi-layer of multi-potent cells, which resembles the appearance of a rudimentary eye (Fig. 1.1C). At the same time as the optic vesicles undergo invagination, the over-lying surface ectoderm also invaginates to produce another vesicle inside the optic cup, which is known as the lens vesicle. This lens vesicle subsequently continues to develop into the eye lens which will focus light onto the retina. The remaining surface ectoderm will go on to form the cornea, where light enters the eye.

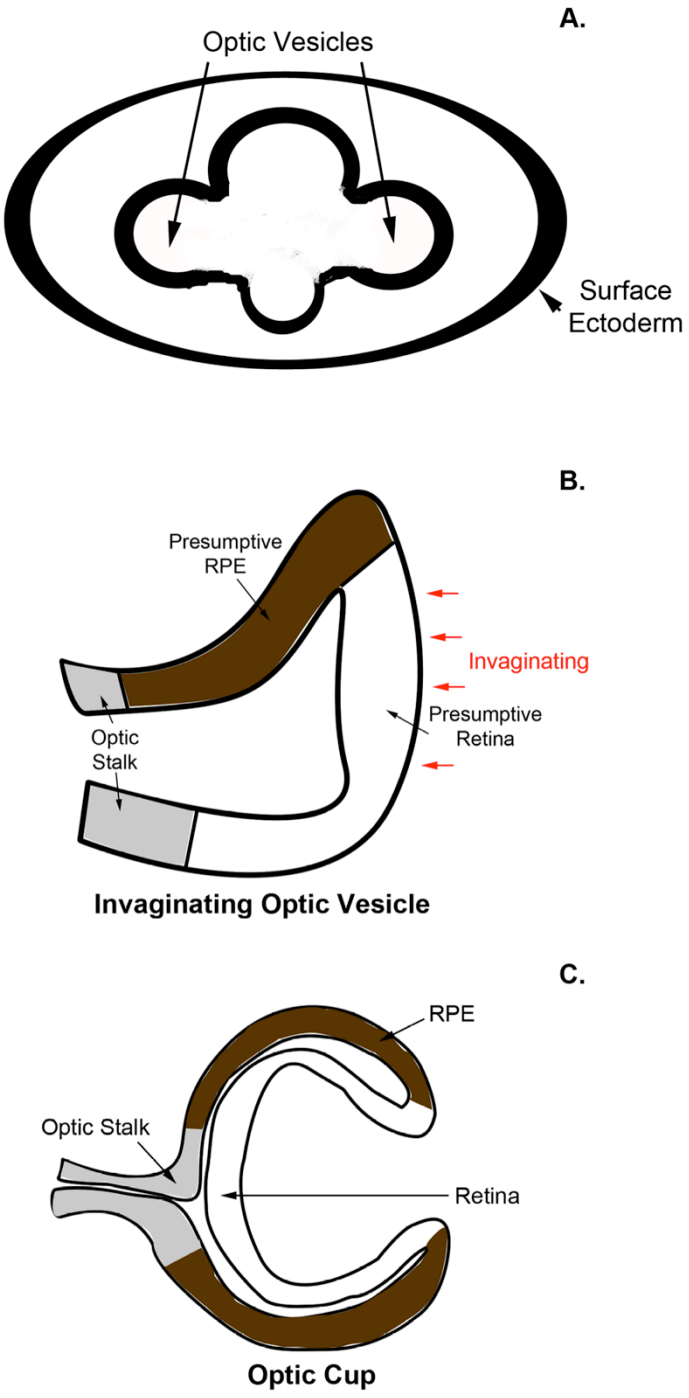


Fig. 1.1
Early eye development
(A) Optic vesicles bud from diencephalon and move towards surface ectoderm. (B) Single-layer of cells in the optic vesicle begins to invaginate. Dorsal cells become RPE, whilst the ventral-most cells become the opptic stalk. Those in-between become the neural retina. (C) Optic cup forms following completion of invagination. Outer layer differentiates as RPE and the inner layer as neural retina.

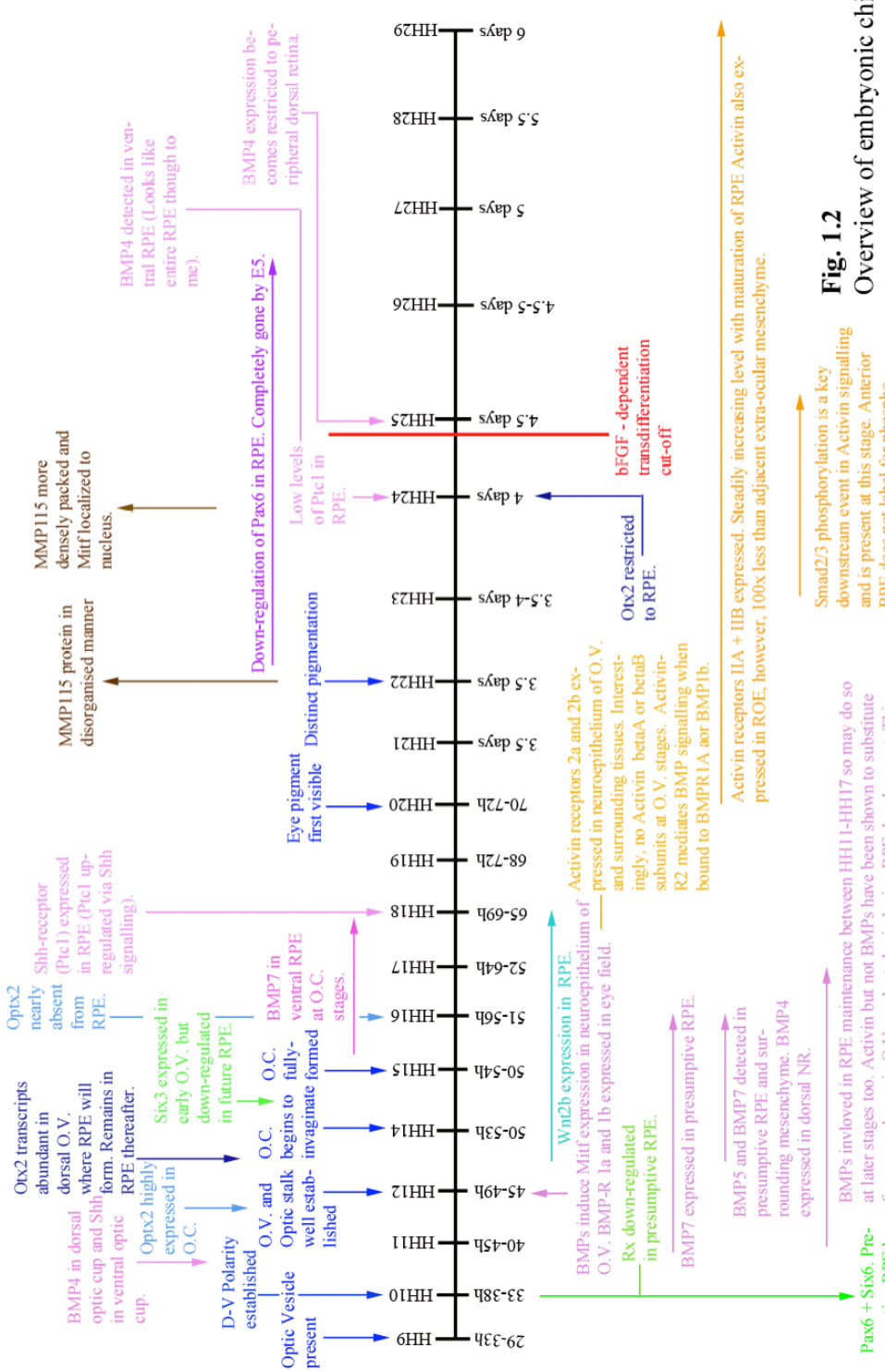


Fig. 1.2
Overview of embryonic chick RPE development

The optic cup stage of development is where the basic cellular patterning in the eye is established (Fuhrmann, 2010). The outer layer of the optic cup consists of a single layer of cells, known as the presumptive RPE (Fig. 1.1C), as a reflection of the fact that these multi-potent cells will eventually become pigmented and form the RPE. The inner layer of the optic cup is known as the presumptive retina, and will eventually proliferate to produce a thickened, multi-layered neuroepithelium that will form the neural retina (Fig. 1.1C). The molecular control of the continuing development of both the RPE and neural retina is reliant upon a complex array of spatio-temporal signaling gradients present within the optic cup. These signaling gradients control a variety of transcription factors that either specify RPE or neural retina differentiation, in an apparently antagonistic fashion.

Perhaps the most crucial signaling pathways that are present in the developing eye are those emanating from the extra-ocular mesenchyme at the back of the eye, as well as those emanating from the lens and surface ectoderm at the front of the eye. It has been shown that transforming growth factor-beta (TGFβ) family signaling molecules, such as activin, are strongly expressed in the extra-ocular mesenchyme and are critical for the patterning and on-going development of the RPE (Fuhrmann et al., 2000b, Sakami et al., 2008). Indeed treatment of developing optic vesicles with activin is able to initiate a fate switch in the developing neural retina cells of cultured optic vesicles. These activin treated vesicles subsequently re-differentiate towards a pigmented RPE cell fate (Fuhrmann et al., 2000b). Conversely, fibroblast growth factors (FGF's) are expressed in the lens and surface ectoderm and are responsible for the initial specification, and on-going development of the neural retina in the inner layer of the optic cup (Pittack et al., 1997, Zhao et al., 2001, Nguyen and Arnheiter, 2000, Desire et al., 1998). Ectopic application of exogenous FGF's in the optic cup has been shown to initiate a fate switch in the RPE cells towards a neuroretinal phenotype, which demonstrates the importance of this signaling pathway in retinal development (Pittack et al., 1991, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg,

1993, Desire et al., 1998). Additionally, inhibition of FGF signaling using oligo-sense nucleotides has been shown to eliminate neural retinal development, with the inner layer of the optic cup instead taking on RPE-like characteristics in a similar manner to eyes treated with ectopic activin (Desire et al., 1998). The presence of both RPE specifying, and neural retina specifying, signaling pathways emanating from opposite sides of the eye suggests the presence of an antagonistic signaling gradient within the eye, which is able to direct the differentiation of the multi-potent cells of both layers of the optic cup towards either and RPE, or neural retinal, fate in a manner dependent upon their localization. The presumptive RPE, being closer to the extraocular mesenchyme, and therefore subject to a higher concentration of activin-like signals, is encouraged to differentiate towards an RPE fate, whereas the presumptive neural retina, which lies in a position closer to the lens and surface ectoderm, is encouraged to follow a neural differentiation pathway, as a consequence of the greater influence of a higher concentration of FGF signaling molecules. The on-going plasticity of the cells of both layers of the optic cup is demonstrated by the fact that both retain the capacity to differentiate into both RPE and neural retinal cells if this carefully balanced signaling gradient is artificially manipulated. Eventually, once the layers of the optic cup have become more mature, there is an apparent loss in the capacity for phenotypic switching, which is a characteristic of more differentiated cells, in contrast with the progenitor characteristics they display in early development. Indeed it has been suggested that a loss in the capacity for RPE cells to respond to FGF signaling is required for full maturation of the RPE, via modulation of the expression of the FGF-receptor, FGF-R1, after the optic cup stage (Spence et al., 2004, Fujiwara, 1994).

These two signaling pathways appear to directly control a number of transcription factor networks that are responsible for the differentiation of both the RPE and the neural retina. Within the RPE, two key transcription factors appear to act at a similar hierachical level in order to regulate the development and maintenance of an RPE phenotype, including the expression of various components of the molecular machinery required for RPE function, as well as pigmentation. These two transcription factors are microphthalmia-associated

transcription factor (Mitf) and orthodenticle homeobox transcription factor 2 (Otx2) (Martinez-Morales et al., 2001, Martinez-Morales et al., 2003, Martinez-Morales et al., 2004, Zuber et al., 2003, Bovolenta et al., 1997, Esumi et al., 2009, Westenskow et al., Larsen et al., 2009, Beby et al., 2010, Fujimura et al., 2009, Muller et al., 2007, Iwakiri et al., 2005, Mochii et al., 1998b, Mochii et al., 1998a, Fuhrmann et al., 2000b, Shibahara et al., 2000, Tsukiji et al., 2009, Planque et al., 2001, Kagiyama et al., 2005, Shibahara et al., 2001, Adijanto et al., 2012, Liu et al., 2009a, Baxter and Pavan, 2003, Bharti et al., 2012). These proteins are able to act synergistically to pattern the RPE domain, and it has been shown that over-expression of Otx2 in neural retinal cells is able to induce a pigmented phenotype in these cells, which underlines its role in specification of the RPE (Martinez-Morales et al., 2003). Both Otx2 and Mitf are also observed to be maintained in later RPE development, which highlights the need for these transcription factors for the maintenance of the RPE phenotype (Bovolenta et al., 1997, Martinez-Morales et al., 2003). Mitf expression appears to be augmented by the activation of activin signaling, which is consistent with the role of both activin signaling, and Mitf, in the specification of RPE cells in the optic cup (Fuhrmann et al., 2000b). Conversely, initial neural retina specification is regulated by another group of transcription factors, namely ceh-10 containing homologue Chx10 and paired box protein 6 (Pax6), which have both been shown to promote retinal differentiation (Azuma et al., 2005a, Azuma et al., 2005b, Oron-Karni et al., 2008, Ashery-Padan and Gruss, 2001, Matsushima et al., 2011, Burmeister et al., 1996, Rowan et al., 2004, Horsford et al., 2005, Chen and Cepko, 2000). Chx10 is expressed in neural retinal progenitors and is responsible for specifying the retinal fate of these cells. Chx10 expression appears to be maintained by FGF signaling emanating from the surface ectoderm as removal of this region in developing embryos results in a loss in the expression of Chx10 in the neural retina, which subsequently expresses Mitf and assumes a pigmented phenotype (Nguyen and Arnheiter, 2000). This increase in Mitf can be suppressed through ectopic addition of FGF's alone, and a consequent maintenance of Chx10 expression is observed (Nguyen and Arnheiter, 2000). It has been demonstrated that FGF suppression of Mitf in the optic cup is dependent on the up-regulation of Chx10, which subsequently inhibits

the expression of Mitf in optic cup progenitor cells (Horsford et al., 2005). A loss in functional Chx10 expression in the developing optic cup leads to both layers of the cup exhibiting an RPE phenotype. This implies that the antagonistic relationship between RPE and neural retina specifying signaling gradients, is reflected in an antagonistic RPE and neural retina specifying relationship between Mitf and Chx10 transcription factors respectively (Nguyen and Arnheiter, 2000, Rowan et al., 2004, Horsford et al., 2005). The involvement of Pax6 in specification of the optic cup is much more complicated, which is unsurprising given its central role in eye development, often being referred to as the 'master eye gene,' due to its involvement in the initial development of the eye, as well as a number of, if not all, the cells of the retina, at some stage in their development (Ashery-Padan and Gruss, 2001, Chow et al., 1999, Zuber et al., 2003, Quinn et al., 1996, Nishina et al., 1999). Pax6 expression is required for both RPE and neural specification, and it appears as though complex regulation of both the expression level of this transcription factor and its modulation of transcription targets dictate progenitor specification in the eye (Bharti et al., 2012, Baumer et al., 2003). A down-regulation of Pax6 in the RPE at a relatively early stage of development is usually associated with the on-going maturation of RPE cells, as well as the commitment of these cells to an RPE phenotype (Spence et al., 2007b). The up-regulation of the transcription factor is usually implicated in an RPE to neural retina fate switch (Azuma et al., 2005a, Spence et al., 2007b, Baumer et al., 2003). However, Pax6, with Pax2, is known to bind and co-activate the expression of RPE transcription factor Mitf. The absence of both these transcription factors in early development leads to development of an ectopic neuroepithelium in the outer layer of the optic cup (Baumer et al., 2003). It has been suggested that Pax6, in the presence of RPE defining factors such as Mitf, acts to augment the RPE phenotype via inhibition of FGF expression and modulation of the Wnt signaling pathway, another pathway known to be important for RPE specification. However, in the presence of neural retina associated factors, Pax6 acts to promote neural retinal development (Bharti et al., 2012). This suggests the presence of a carefully regulated feedback loop that governs the patterning of the optic vesicle.

A number of other signaling pathways have been involved in the patterning of the optic cup, both via establishment of the RPE and neural retina domains, as well as distinct dorso-ventral patterning of the retina. One of these is the aforementioned Wnt signaling pathway, which has been implicated in the specification of the dorsal retina domain, as well as the RPE (Bharti et al., 2012, Burke, 2008, Westenskow et al., Fujimura et al., 2009, Veien et al., 2008). Transcriptional inactivation of the canonical Wnt/β-catenin signaling pathway results in a loss of the dorsal RPE phenotype, which is instead replaced by a neural retinal identity, indicating the important role that this signaling pathway plays in RPE development (Fujimura et al., 2009). Additionally, the FGF and Dkk signaling pathways are able to initiate a neural retinal program of differentiation via inhibition of the Wnt/β-catenin signaling pathway (Bharti et al., 2012).

Sonic hedgehog (Shh) and bone morphogenic proteins (BMP's) have also both been implicated in RPE development, as well as the dorso-ventral patterning of the retina. Shh is primarily expressed in the ventral retina, where it promotes the expression of ventralizing factors such as Pax2, required for specification of the optic stalk domain, as well as ventral anterior homeobox protein c (cVax) (Zhang and Yang, 2001). Shh also restricts the expression of Pax6 in this region. Correspondingly, the dorsal retina primarily expresses BMP family members, which promote the specification of the dorsal features of the retina, including expression of dorsal specification factors like T-box transcription factor 2/3/5 (tbx2/3/5), and inhibit the expression of Shh in this region (Zhang and Yang, 2001, Sasagawa et al., 2002). Mis-expression of a natural inhibitor of BMP signaling, noggin, results in the expansion of the ventral domain into the region normally occupied by dorsal specification factors, in a similar manner to that observed via ectopic expression of Shh in the eye (Zhang and Yang, 2001, Sasagawa et al., 2002), whereas inhibition of Shh activity leads to an increase in the domain expressing dorsal factors, including BMP. Taken together, this suggests the existence of a dorso-ventral signaling gradient between BMP and Shh respectively, which act to pattern the dorso-ventral characteristics of the retina through a Shh-

dependent restriction of the BMP signaling domain. In addition to dorso-ventral patterning, both Shh and BMP appear to be involved in the augmentation of the RPE phenotype in the ventral and dorsal domains of the optic cup, respectively (Zhang and Yang, 2001, Spence et al., 2007a, Muller et al., 2007). Inhibition of either of these signaling pathways leads to a down-regulation in the expression of RPE markers such as Mitf and Otx2 within the RPE layer, and a subsequently transdifferentiation towards a neuroretinal phenotype, providing further evidence for their involvement in the maintenance of the RPE phenotype. It has been suggested that BMP signaling may also interact with Wnt/β-catenin signaling to initiate dorsal patterning and RPE specification in the dorsal domain, given the similar effects that inhibition of either pathway appears to elicit during eye development (Veien et al., 2008).

Additional transcription factors implicated in the development of the RPE include: the activating enhancer-binding protein 2 alpha (AP2α) transcription factor (West-Mays et al., 1999), as well as the growth arrest specific gene 1 (Gas1)(Lee et al., 2001). Loss of function mutants for each of these factors results in the loss of RPE specification, with a subsequent conversion to a neural retina phenotype in the outer layer of the optic cup.

Despite the complex regulation surrounding the development of both the RPE and neural retina following optic cup invagination, ultimately, the final fate decision for the cells of the optic cup lies with the dominant, micro-environmental signaling pathway, the outcome of which is determined by the localization of a particular progenitor cell, or layer of progenitor cells, in the antagonistic signaling gradients found to be present during eye development.

1.3 Age-related Macular Degeneration (AMD):

Age-related macular degeneration is the most common form of blindness in the developed world, with approximately 15 million sufferers in the United States alone. This number is expected to rise as the average life expectancy continues to increase, and

subsequently degenerative diseases become more common in an aged population. As the name suggests, the disease is normally observed in patients aged 50 and over (Wang et al., 2010), and often involves gradual deterioration of the central vision associated with the macular, which is critical for fine vision in common tasks such as reading, driving, and facial recognition. There are two known forms of the disease: exudative (often known as 'wet AMD'), and non-exudative (often known as 'dry AMD'). Both forms of the disease result in a loss of central vision as a consequence of the degeneration of retinal photoreceptors, cells in the eye which are responsible for sensing light, which subsequently means that light can no longer be detected within the macular region. Our understanding of the causes of the disease are limited, however, there are distinct differences between the two aforementioned forms of AMD (Klein et al., 2004, Nowak, 2006, Young, 1987).

The dry form of AMD involves the build up of yellow, lipid/protein, waste deposits between the retinal pigment epithelium (RPE) and the choroid, which houses the extensive retinal vasculature. These deposits are referred to as 'drusen' and it is currently unclear as to why they are produced. Drusen are found in healthy eyes later in life, however, they are much greater in number over the macular region in diseased eyes, where they appear to affect the proper function of the RPE, perhaps indicating that the RPE are not functioning correctly. The RPE performs a number of important functions to support the retina, including phagocytosis, and re-cycling of retinal waste during the process of phototransduction. Disruption of these roles means that the RPE is no longer able to support the retina, and the outer layer of the retina containing the photoreceptors begins the degenerate (Curcio et al., 1996). This outcome has been demonstrated in an animal model of retinal degeneration, the Royal College of Surgeon's (RCS) rat (Li and Turner, 1988, Seaton et al., 1994, Lund et al., 2001, Coffey et al., 2002, Wang et al., 2005, Carr et al., 2009c, Lund et al., 2006a, Lopez et al., 1989, Little et al., 1998, Girman et al., 2003, Gias et al., 2007, Lund et al., 2006b), which contains a c-mer proto-oncogene tyrosine kinase (MERTK) mutation within the RPE, preventing phagocytosis of retinal waste from taking place (D'Cruz et al., 2000). In this

instance, the photoreceptor layer is observed to degenerate over time, as in patients with AMD. There are currently very few effective treatments available for this form of the disease.

The exudative form of AMD also involves the loss of photoreceptors, however, the pathology of this form is different from that described for dry AMD. Wet AMD involves a break down in the physical and chemical blood:retinal barrier that the RPE and Bruch's membrane provide to prevent neo-vascularization of the retina, as a result of the degeneration of the RPE. Without the barrier that the RPE provides, the vasculature is able to extend into the retinal layers. These new fragile vessels often leak, resulting in a bleed within the macular region. This neo-vascularization results in a loss of the critical retinal architecture, and subsequently a degeneration of retinal photoreceptors. There are several clinical treatments available, which are designed to limit the progression of this form of AMD. The most common of these treatments involves the administration of anti-angiogenic factors directly into the eye. These drugs, targeted to the anti-vascular endothelial growth factor (VEGF) include *lucentis*, which prevent the progression of new vasculature growth into the retina. Other treatments involve laser photo-coagulation, which can prevent new vasculature being formed, as well as slow the progression of neovascularization in the retina.

In addition to pharmacological and photocoagulation techniques, several different attempts at surgical RPE transplantation have undertaken in order to attempt to restore the function of the RPE, and therefore prevent the loss of vision (for review see (da Cruz et al., 2007).

1.4 Autologous RPE transplantation:

One approach employed in an attempt to halt the degeneration of photoreceptors in AMD, is to replace the damaged RPE cells found under the macular region of the retina with functional RPE cells from the peripheral region of the patient's own eye. By doing so, the central retina is therefore in contact with functional RPE cells that may be healthier than those in the macular, and therefore can provide all the normal functions necessary to preserve the

retina, and subsequently rescue vision. This procedure is able to halt the progressive degeneration of retinal photoreceptors, which is a characteristic of AMD. However, the procedure is difficult to perform and can take multiple hours and a number of operations to first harvest the patient's own cells, then re-position them beneath the diseased macula (Siqueira, 2009, Peyman et al., 1991, Binder et al., 2004).

1.5 Macula Translocation:

Another technique relies upon a similar principle, taking advantage of the fact that the healthy peripheral RPE cells are likely to display better function than those cells beneath the macula region. In this instance, RPE cells are not harvested from the patient's peripheral eye, but instead the retina is detached from the under-lying RPE as an entire sheet and rotated to reposition the macular over healthy RPE,. As a result of this treatment, patients again cease to exhibit retinal degeneration and observe a consequential rescue of vision. This procedure does not represent a true RPE transplantation strategy, but is effectively a functional RPE transplantation, given that the re-positioned macula is now in contact with a different, functional region of RPE not affected by the progression of AMD. Given the positive outcome that this technique has had on patient sight, it currently represents the gold-standard in RPE transplantation efficacy for on-going development of new strategies for RPE replacement (Lai et al., 2002). Despite this, the procedure is not widely practiced owing to the high level of expertise required, and the difficulty of the operation itself. Therefore, despite encouraging evidence that RPE transplantation may provide an effective cure for AMD, further development of RPE transplantation strategies will be required in order for the procedure to become routine.

1.6 HESC-RPE Transplantation:

A number of different sources of RPE cells have been utilized for transplantation into animal models/patients with AMD, however, a number of problems have been associated with many of these sources. These problems are usually associated with the sustainability in the supply, as with primary RPE sources, or the functionality of the cells, as with established, immortalized cell lines (reviewed by (da Cruz et al., 2007)). However, recent development in this field has employed the use of human embryonic stem cell-derived RPE cells (HESC-RPE), which represent a sustainable source of RPE cells for transplantation, as well as having been shown to exhibit a robust, functional RPE phenotype in animal models of AMD (Lund et al., 2006b, Vugler et al., 2008, Klimanskaya et al., 2004, Lu et al., 2009, Idelson et al., 2009). Several groups are utilizing HESC-RPE, via sub-retinal injection of cell suspensions or surgical transplantation of intact monolayers, in order to attempt to prevent the degeneration of photoreceptors in patients with diseases such as AMD and Staargart's disease (Schwartz et al., 2012). Despite encouraging evidence subretinal injection of HESC-RPE may rescue the vision of some patients suffering from these types of blindness, this approach is designed to halt further degeneration of photoreceptors following surgical intervention, and therefore is only suitable for patients at an early stage of degeneration who still retain a large proportion of their sight. This technique would therefore not be suitable for later stage patients who have already lost the majority of their macula photoreceptors, and subsequently most of their central vision. This has encouraged research into the potential for photoreceptor transplantation for diseases where these cells are lost.

1.7 Photoreceptor transplantation:

There is great hope for cell transplantation therapies for a number of retinal diseases, including production of new ganglion cells from Muller glia for treatment of glaucoma, and transplantation of retinal progenitor cells for treatment of other retinal degenerative diseases

(Hollborn et al., 2011, Singhal et al., 2012, Zhao et al., 2008, Seiler and Aramant, 2005, Qiu et al., 2005, Klassen et al., 2004, Klassen, 2006). Additionally, several studies have reported that photoreceptor transplantation may provide a solution for the repair of damaged retinas in patients with a number of retinal degenerative diseases (Gust and Reh, 2011, MacLaren et al., 2006, Singh et al., 2013, Pearson et al., 2012). It has been reported that immature rod photoreceptor cells only are able to integrate into the mouse retina following harvesting from embryos at specific stages of rod photoreceptor cell development (MacLaren et al., 2006). These post-mitotic rods have been shown to express both cone-rod homeobox gene (CRX) and neural retina leucine zipper (NRL) proteins, which have both been implicated in photoreceptor differentiation and development (MacLaren et al., 2006, Rutherford et al., 2004, Jomary and Jones, 2008, Oron-Karni et al., 2008, Peng and Chen, 2007, Swaroop et al., 1999, Kimura et al., 2000, Glubrecht et al., 2009, Hennig et al., 2008, Hendrickson et al., 2008, Khanna et al., 2006, Gust and Reh, 2011). MacLaren et al (2006) and Pearson et al (2012) suggest that post-mitotic rods from developmental stage P4-8 can integrate into the adult host retina, continue their differentiation, and improve vision in a mouse model, Gnat1$^{-/-}$, which lacks rod function (Pearson et al., 2012, MacLaren et al., 2006). However, in contrast to the evidence that the ability to integrate is limited to immature, post-mitotic photoreceptors, another study has suggested that the capacity for integration of photoreceptor cells is not only limited to early stages of development, but can actually extend to photoreceptor cells from adult donors (Gust and Reh, 2011). The authors suggest that the numbers of cells that can integrate into the host retina is equivalent at both immature, post-mitotic stages, and adult stages, in successful transplants. However, the apparent difference in the capacity for integration actually reflects the survivability of the cells following dissociation prior to injection. It is suggested that fully-differentiated photoreceptor cells from adult donors have a higher transplant failure, which could possibly be misinterpreted as a lower propensity for integration (Gust and Reh, 2011).

Although encouraging, the above studies focused on the capacity for donor photoreceptors to integrate and restore vision in hosts that retained an intact retinal architecture, which is

unreflective of the retinal environment in diseased eyes which have undergone, a degree of, or complete, degeneration of the outer retinal layer containing the photoreceptors. It was necessary to confirm whether transplanted photoreceptors could integrate into a retina with widespread alterations in the structural and circuitry architecture that are associated with retinal degeneration. Encouragingly, transplantation of post-mitotic rod progenitors were observed to have the capacity to reconstitute the rods in the photoreceptor layer in a late stage mouse model of retinisis pigmentosa, at a point where untreated animals would normally lack the presence of rods in the retina (Singh et al., 2013). Additionally, integrated cells were able to restore a level of vision in these animals as assayed by pupillary light response (PLR), behavioural light aversion, and optical imaging (OI), which suggests that the host mice can detect light, however, the quality of visual information that they are able to observe remains unclear.

1.8 Sources of photoreceptors for transplantation:

There are a number of current issues with employing a photoreceptor transplantation approach to treatment of patients who have experienced retinal degeneration, not least whether the new cells can confer useful vision in the recipient patients. However, one of the major problems with this approach is the lack of a sustainable source of photoreceptors for transplantation. Some studies have employed the use of human retinal photoreceptor precursors with limited improvement in vision, if any (Radtke et al., 2008, Humayun et al., 2000). The problem with this approach, in addition to the limited efficacy of the approach reported in these studies, is the sustainability of the supply of the appropriate human retinal tissue for on-going development of the technique, as well as the required quantities of foetal tissue from specific developmental stages needed to treat a large clinical population. Therefore, human fetal tissue is not particularly suitable for transplantation.
In light of this, many groups are now attempting to utilize the relatively new field of regenerative medicine and stem cells in order to produce a sustainable supply of

photoreceptors for transdifferentiation, in much the same way as has been demonstrated for HESC-RPE. A number of authors have reported protocols which claim to produce retinal cell types, in some cases including rod photoreceptors, from both HESC and induced-pluripotent stem cells (iPS cells), which could possibly be used for transplantation into patients suffering from retinal degenerative diseases (Meyer et al., 2011, Meyer et al., 2009, Osakada et al., 2008, Osakada et al., 2009, Hirami et al., 2009, Eiraku and Sasai, 2012a, Eiraku and Sasai, 2012b). Different reports describe various protocols for the generation of cells exhibiting a retinal photoreceptor phenotype, with a number of them requiring at least a period of 3D, non-adherent culture, in addition to treatment with various growth factors. Some of these involve differentiation of stem cells directly towards a photoreceptor phenotype, often in a disorganized, heterogenous mass of differentiating progenitor cells, however, more recent accounts have demonstrated the ability to produce intact, optic cup structures, which contain cells with both a number of retinal cell phenotypes, in addition to RPE cells (Eiraku et al., 2012, Eiraku and Sasai, 2012b, Eiraku and Sasai, 2012a). Despite reports of the differentiation of cells expressing retinal markers, including rod marker rhodopsin, no study has yet demonstrated the differentiation of functional photoreceptors from direct differentiation of various stem cell technologies. Additionally, many of these protocols have proved difficult to replicate, possibly as a result of the complex nature of the differentiation process, the cell source employed, as well as the likelihood of a very low % yield of retinal cell types in these cultures. Despite encouraging and on-going research in these areas, it is possible that a slightly different approach to differentiation might yet yield more positive results.

1.9 Transdifferentiation of the RPE:

A particularly interesting feature of RPE cells with regard to the production of novel retinal cell types is a phenomenon that has been described in a number of different species known as RPE transdifferentiation. This is a process whereby the RPE is able to re-

differentiate towards either a neuro-retinal, or lens phenotype in response to a number of different environmental cues (Vugler et al., 2007, Reh and Pittack, 1995, Zhao et al., 1997, Araki, 2007). This phenomenon has been reported in amphibians such as *urodeles* (newts)(Susaki and Chiba, 2007, Chiba et al., 2006b, Ikegami et al., 2002, Araki, 2007, Kodama and Eguchi, 1995, Sakami et al., 2005, Mitsuda et al., 2005, Grigorian and Mitashov, 1985, Kaneko and Chiba, 2008, Avdonin et al., 2008, Nakamura and Chiba, 2007, Eguchi, 1988, Kuriyama et al., 2009a, Chiba et al., 2006a, Tsonis et al., 2004) and *xenopus* (African clawed toad)(Yoshii et al., 2007a, Vergara and Del Rio-Tsonis, 2009, Arresta et al., 2005, Sakaguchi et al., 1997), birds such as chickens and quails (Opas and Dziak, 1994b, Liang et al., 2006a, Galy et al., 2002, Yan and Wang, 2000b, Yan and Wang, 2000a, Ma et al., 2004b, Spence et al., 2007b, Spence et al., 2004, Azuma et al., 2005a, Hyuga et al., 1993, Coulombre, 1981, Sakami et al., 2008, Park and Hollenberg, 1989, Pittack et al., 1991, Reh et al., 1991, Matsuo et al., 1998, Zhou and Opas, 1994, Park and Hollenberg, 1991, Toy et al., 1998, Mochii et al., 1998b, Mochii et al., 1998a, Mochii et al., 1988, Itoh and Eguchi, 1986, Eguchi, 1986, Eguchi, 1988, Okada et al., 1983, Okada et al., 1982, Okada and Yasuda, 1993, Yasuda et al., 1981, Rowan et al., 2004, Ma et al., 2009, Ma et al., 2004a, Araki et al., 2002, Liang et al., 2006b, Fischer and Reh, 2001, Opas and Dziak, 1994a, Wang and Yan, 2012, Coulombre and Coulombre, 1970, Coulombre and Coulombre, 1965), as well as mammalian species including both rats and mice (Zhao et al., 1995, Sakami et al., 2008).

Unlike most reported forms of RPE transdifferentiation, newt RPE transdifferentiation occurs during the entire life of the animal, which is subsequently able to regenerate its retina following removal or damage, without the need for exogenous intervention. The regenerated retina resulting from RPE transdifferentiation displays all the expected retinal markers and cell types and is correctly organized with the photoreceptor layer immediately adjacent to the RPE monolayer, and the ganglion cell layer at the vitreal surface of the retina (Chiba et al., 2006b, Nakamura and Chiba, 2007, Kuriyama et al., 2009a, Kaneko and Chiba, 2009). As a result the retina is fully functional and the animal can once again see. Crucially, this instance of RPE transdifferentiation produces a novel, intact, RPE monolayer

in addition to a novel retina, which allows the new retina to function properly, and implies that a pure regenerative event has occurred.

In contrast, the capacity for RPE transdifferentiation reported in other species does not extend to the adult, but instead is restricted to comparatively early stages of development, post-pigmentation of the RPE (Park and Hollenberg, 1989, Pittack et al., 1991, Pittack et al., 1997, Sakami et al., 2008, Zhao et al., 1995). In addition, the developing retina resulting from RPE transdifferentiation is inverted in comparison to the native retina, with the ganglion cell layer in the proximal portion of the neuroepithelium, and the outer nuclear layer being found in the distal portion (Sakami et al., 2008, Opas and Dziak, 1994a). Additionally, unlike newt retinal regeneration, the RPE monolayer is not regenerated in these models of RPE transdifferentiation. As a result of the lack of RPE following transdifferentiation, in addition to the inverted nature of the resulting retina, the novel retina is not physiologically functional.

The phenomenon has been replicated both *in vivo* and *in vitro* for most, if not all the species in which it has been described, and there are a number of similarities in both the induction of the neuronal phenotype, and progression of its development, across these species. The major cue which has been implicated with the onset of transdifferentiation is the exposure to higher levels of fibroblast growth factors (FGF's), namely basic FGF (bFGF/FGF2) (Park and Hollenberg, 1989, Opas and Dziak, 1994b, Yoshii et al., 2007b, Spence et al., 2007b, Spence et al., 2004, Sakami et al., 2008, Pittack et al., 1991, Sakaguchi et al., 1997, Reh et al., 1991, Kuriyama et al., 2009b, Bharti et al., 2012) and a number of other FGF family members, including FGF8 (Vogel-Hopker et al., 2000), FGF9 (Zhao et al., 2001) and FGF15 (Bharti et al., 2012). The onset of transdifferentiation appears to mimic the normal development of the multi-potent cells of the optic cup, where FGF, released from the surface ectoderm and lens, down-regulates the expression of RPE differentiation machinery in the multi-potent cells of the optic cup, including key RPE transcription factors, Mitf and Otx2, which up-regulate transcription factors associated with retinal development, such as Pax6 and Chx10 (Nguyen and Arnheiter, 2000, Ahmad et al., 2000, Horsford et al., 2005, Spence et al., 2007a, Spence et al., 2007b, Sakami et al., 2005). This initiates proliferation and subsequently

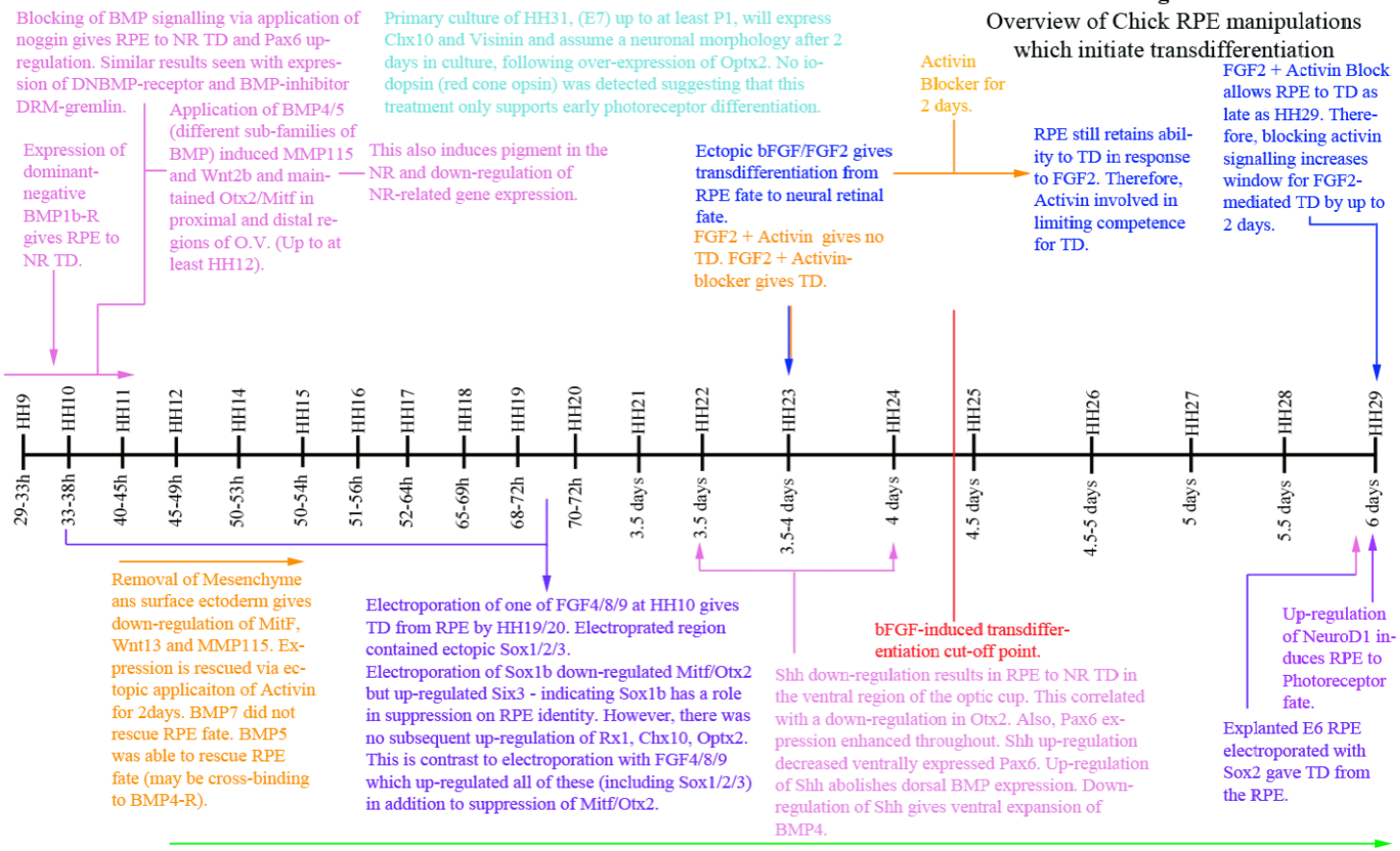
differentiation of the initial multi-potent cell population to produce a retinal neuroepithelium. Indeed FGF's in transdifferentiation have been demonstrated to initiate a down-regulation of RPE markers such as Mitf, and initiate the expression of neural retinal markers like Pax6 in a bFGF/FGFR1/MEK/Erk/Pax6-dependent manner (Spence et al., 2007b, Pacheco-Dominguez et al., 2008, Mitsuda et al., 2005, Galy et al., 2002, Susaki and Chiba, 2007). Additionally, ectopic expression of Pax6 is itself sufficient for the induction of transdifferentiation in the chick model of the phenomenon, which would suggest that Pax6 operates as a master regulator of transdifferentiation (Azuma et al., 2005a). Oncogene Ras has also been implicated in the signaling pathway responsible for the induction of transdifferentiation, which could point to a role in the initial RPEproliferation required for transdifferentiation to occur (Dutt et al., 1993, Zhao et al., 2001). Interestingly, some authors have suggested that bFGF does not act as the initiator of transdifferentiation in the newt model of the phenomenon (Susaki and Chiba, 2007, Kuriyama et al., 2009a), which appears to be the case in other models such as the chick (Park and Hollenberg, 1989, Pittack et al., 1997, Pittack et al., 1991, Opas and Dziak, 1994b). These reports suggest that although a bFGF signaling cascade is heavily involved in the early stages of transdifferentiation, bFGF does not initiate Pax6 expression in the latent newt RPE, but instead acts on Pax6 positive RPE cells, which up-regulate the transcription factor via an altered interaction with the choroid in the absence of bFGF, in an *in vitro* organ culture system. The authors note that the initiation of Pax6 expression in newt RPE upon removal from the choroid is reversible if the cells are re-attached (Kuriyama et al., 2009b). However, bFGF is required to maintain the Pax6 expression in these cells, and also to drive transdifferentiation. Another study demonstrated that the newt RPE cells require contact with the choroid to undergo transdifferentiation, and did not undergo the phenomenon when it was removed (Mitsuda et al., 2005). The effect of the choroid was found to be restored when the RPE monolayer and choroid were separated by a filter membrane, implying that the factor(s) responsible were diffusible, and also that inhibition of the FGF signaling pathway inhibited transdifferentiation in the presence of the choroid, once again highlighting the central role this signaling pathway plays in

transdifferentiation (Mitsuda et al., 2005). Indeed a number of reports have described the important role of the extracellular matrix in transdifferentiation in both the newt (Reh et al., 1987) and the chick (Opas and Dziak, 1994b) models of the phenomenon. It is possible that RPE cells must first express Pax6 in order for bFGF to be effective in initiating transdifferentiation. bFGF treatment of RPE cells is known to induce Sox2 expression, and each of bFGF and Sox2 subsequently form a positive feedback loop to enhance one another's expression (Lin et al., 2009). Sox2 is an important transcription factor known to be crucial for neuronal competence in the developing retina (Matsushima et al., 2011), and is up-regulated early in transdifferentiation (Lin et al., 2009). Sox2 is able to act in synergy with Pax6, via a physical complex, to modulate both the expression of Pax6, and its transcriptional targets (Aota et al., 2003, Cvekl et al., 2004, Lin et al., 2009, Matsushima et al., 2011). Given its central role in the modification of Pax6 function, it is possible that the combined action of Sox2 and Pax6 in the early stages of transdifferentiation requires a latent Pax6 expression in the RPE prior to bFGF treatment to drive transdifferentiation in the newt. If this is the case, this may account for the fact that newt RPE can transdifferentiate throughout the life of the animal, whereas other species only transdifferentiate at early embryonic stages in response to bFGF, but become restricted beyond a particular stage of development. Interestingly, the reported loss in potential for transdifferentiation in response to bFGF coincides with a reported loss in the expression of Pax6 within the RPE of the embryonic chick (approximately E5/HH25) (Pittack et al., 1997, Sakami et al., 2008). This stage also corresponds to a equivalent stage of development in the rat (E14.5)(Zhao et al., 1995, Butler, 1987). This would support the idea that maintenance of Pax6 expression is required for exogenous bFGF to drive transdifferentiation, and that the loss in the capacity to respond to bFGF is evolutionarily conserved between mammals and avians. It remains unclear what mechanism is able to restrict to action of bFGF at these stages, however, given that the nature of transdifferentiation appears to reflect the early development of the optic cup, it is likely that factors responsible for the augmentation of an RPE phenotype are responsible. It is likely that these mechanisms are able to negatively modulate the expression of Pax6, as well as inhibit

the bFGF signaling cascade, given that Pax6 transfer alone is reported to initiate transdifferentiation of the RPE at much later stages than that reported for bFGF treatment alone (Azuma et al., 2005a).

In addition to FGF's, Sox2 and Pax6, a number of other factors have been associated with the induction of a neural phenotype in RPE cells. Many of these include inhibitors of RPE development such as cyclopamine and noggin, which inhibit Shh and BMPs respectively. As a result, these inhibitors guide the RPE along the default pathway towards a neuroepithelial fate (Muller et al., 2007, Spence et al., 2007a). In addition, ectopic expression of a number of other factors in RPE has been shown to initiate neuronal differentiation. Among these, NeuroD, a transcription factor associated with photoreceptor development was able to induce a neuronal phenotype (Ma et al., 2004b), as well as the expression of photoreceptor markers including visinin and red opsin, when ectopically expressed in dissociated E6 RPE cells (Yan and Wang, 2000b, Yan and Wang, 2000a). These cells were able to integrate into the host retina following sub-retinal transplantation into E5-7 chick embryos, exhibiting neuronal processes which extended out from the cell body, and appeared to make synaptic connections with adjacent retinal cells (Liang et al., 2006a). Addition of bFGF to these cultures did not significantly increase the expression of photoreceptor markers in the RPE cells, however, bFGF could induce some neuronal marker, RA4 antigen expression, without the presence of a neuronal morphology or additional photoreceptor marker expression. A similar effect was observed when the eye field transcription factor, optic six gene 2 (optx2), was ectopically expressed in both embryonic and mature chicken RPE cells (Toy et al., 1998). As a result, RPE cells expressed retinal and photoreceptor markers and began to exhibit a neuronal morphology, implying that these cells may have undergone transdifferentiation. This demonstrates the on-going plasticity of RPE cells at slightly later stages of development (An overview of previous transdifferentiation experiments in the chick is displayed in Fig. 1.3).

Fig. 1.3
Overview of Chick RPE manipulations which initiate transdifferentiation



Despite some reports of the expression of neuronal markers in dissociated, adherent cultures of RPE cells, retinal markers of photoreceptor specification have only been reported via ectopic expression of master transcription factor regulators, such as NeuroD, and not through treatment with exogenous growth factors *in vitro*. Additionally, it is still unclear whether the resulting retina-like cells have undergone a true phenotypic change, or have simply begun to express various markers of retinal lineage, without having undergone full re-programming. Given the complex nature of the 3-dimensional, spatio-temporal signaling required for normal retinal development, it is likely that these cells have not developed in as complete a manner as their native counterparts. Even transdifferentiating newt RPE cells do not appear to produce all the retinal cell types in a pure, monolayer, culture system (Mitsuda et al., 2005, Susaki and Chiba, 2007).

Classical bFGF studies of transdifferentiation, which produce in a fully formed neuroepithelium from transdifferentiated RPE cells, more closely resemble the developing native retina, albeit with an inverted phenotype (Pittack et al., 1991, Opas and Dziak, 1994b, Sakami et al., 2008). It is likely that the transdifferentiated neuroepithelium is more likely to reflect the normal developmental program of the native retina given its intact, structural nature, with a less disrupted spatio-temporal signaling environment given the more natural distribution of the different retinal cell types. Intriguingly, it has been reported that the capacity for RPE transdifferentiation is modulated by the mechanical properties of the substratum (Opas and Dziak, 1994b), in addition to the composition of the substratum/extracellular matrix as described earlier. The reason for this is unclear, however, the most robust classical transdifferentiation in response to bFGF is produced when intact sheets of RPE explants are cultured in non-adherent conditions (Sakami et al., 2008, Pittack et al., 1991, Sakaguchi et al., 1997). In order to observe transdifferentiation, it is critical that RPE explants are cultured as intact sheets, much like those observed to undergo the phenomenon *in vivo*, in the presence of bFGF (Pittack et al., 1991). It is unclear why this is the case, however, it may be that the non-adherent culture system allows flexible migration of

transdifferentiating RPE cells to produce a thickened neuroepithelium. Consistent with this idea, explanted monolayers of newt RPE treated with bFGF are able to undergo full transdifferentiation, producing a retinal neuroepithelium containing various retinal cell types, when the RPE is overlaid by a gel matrix that provides a scaffold for transdifferentiating cells (Kuriyama et al., 2009a). Dissociated RPE cells did not undergo transdifferentiation, with no neuronal marker expression or morphology observed in bFGF treated cultures, despite de-pigmentation and proliferation of the RPE cells (Pittack et al., 1991). It is likely that this de-pigmentation was a result of de-differentiation of the RPE cells, rather than transdifferentiation, which is a feature of RPE undergoing epithelial-mesenchymal transition (EMT) upon dissociation (Liu et al., 2009b).

1.10 Evidence for transdifferentiation of human RPE towards a neuronal phenotype:

A degree of plasticity of the RPE phenotype appears to be evolutionarily conserved across a number of very different species. This has also been found to be true of classical transdifferentiation in response to bFGF, which produces an intact, retina neuroepithelium that expresses developing markers of various retinal cells types. It is therefore reasonable to assume that this phenotypic plasticity may be conserved in human RPE cells, at least at early stages of development like most of the species in which transdifferentiation has been described.

Indeed limited transdifferentiation has been reported in spontaneously immortalized, human RPE cell lines such as ARPE19, in response to retinoic acid, and a synthetic analogue of retinoic acid, known as fenretinide (Carr et al., Chen et al., 2006, Chen et al., 2003). These authors have reported that RPE cells treated with these compounds are observed to lose their characteristic epithelial morphology, and instead exhibit a neuronal morphology, with processes extending out from the cell body. In addition, RPE markers such as Otx2 are down-regulated in treated cultures when compared with DMSO-treated controls, with a subsequent increase in retinal cell markers, Pax6, Sox2, Nrl, and blue opsin (OPN1lw) in fenretinide

treated cells (Carr et al., Chen et al., 2006, Chen et al., 2003). However, some retinal markers were also observed in negative control cultures of RPE cell lines, which may suggest that retinoic acid does not actively induce neuronal differentiation, but instead enhances existing neuronal tendencies in these cell lines. It is possible that in down-regulating an RPE master regulator, such as Otx2 (Simeone et al., 1995), retinoic acid treatment is able to cause the RPE cell to de-differentiate further (Martinez-Morales et al., 2003, Martinez-Morales et al., 2004, Esumi et al., 2009, Westenskow et al., Takeda et al., 2003, Beby et al., 2010). As a result, the cells may then begin to express retinal markers as a reflection of their plastic, multi-potent heritage; the default differentiation pathway becoming retina-like in the absence of RPE differentiation cues. Interestingly, the neural phenotype promoting effects of retinoic acid appear to employ at least a portion of the FGF signaling cascade, as the effects of retinoic acid are observed to act via the same downstream effector as FGF, Erk, in addition to being inhibited by MEK inhibitor, U0126 (Samuel et al., 2008, Spence et al., 2007b, Susaki and Chiba, 2007, Pacheco-Dominguez et al., 2008, Galy et al., 2002). This may suggest that retinoic acid is able to activate part of the signaling mechanism required for the induction of transdifferentiation. It may be that other portions of the FGF cascade need to be activated for all retinal cell types to transdifferentiate from RPE cells, however, the lack of all retinal cell types may also reflect a two-dimensional culture system not capable of full retinal development.

Despite encouraging evidence that human RPE cells can undergo limited neuronal differentiation, it still remains unclear whether this is true transdifferentiation, and whether or not retinoic acid would elicit similar effects on RPE cells that do not already express a number of retinal markers. Clearly it does not resemble the type of classical transdifferentiation described above, where RPE cells give way to a relatively well developed retinal neuroepithelium, however, this does demonstrate that human RPE cells still display at least some potential for plasticity. This is encouraging for on-going studies into the potential for human RPE cell transdifferentiation.

1.11 Overview of experimental chapters:

- Chapter 3

An embryonic chick model of transdifferentiation was replicated and characterized in a humanized culture system, to ascertain whether the chick model of the phenomenon was an appropriate comparison for human RPE cells. This included the analysis of retinal marker expression in both developing chick retina, and transdifferentiated neuroepithelium, as well as the capacity for transdifferentiation in chick RPE at different developmental stages.

- Chapter 4

Signaling pathways: Shh and BMP which are associated with RPE phenotype augmentation were assessed for their ability to modulate the expression of Pax6 in developing embryonic chick RPE cells. These pathways were also tested for their ability to inhibit transdifferentiation of embryonic chick RPE in response to bFGF, through co-culture of bFGF with both BMPs and Shh respectively. This was an attempt to identify the signaling mechanisms responsible for the developmental restriction in the capacity for transdifferentiation in response to bFGF observed in later stage embryonic chick RPE.

- Chapter 5

Human fetal tissue sections at different developmental stages were analysed for various markers associated with both retinal and RPE development. Subsequently, primary human fetal RPE tissue at different developmental stages was assessed for its capacity to undergo transdifferentiation in response to bFGF at the earliest stages of development available, comparable to that observed in the chick model of transdifferentiation.

- Chapter 6

HESC-RPE cells were analysed for the expression of important markers associated with transdifferentiation. Additionally, the capacity for transdifferentiation of HESC-RPE cells in response to bFGF was assessed, using the same, established assay. HESC-RPE cells were also assessed for their potential to transdifferentiate in response to bFGF when the activin signaling pathway, known to augment the RPE phenotype, was inhibited.

Chapter 2 – General materials & methods

2.1 Developmental analysis of retinal development:

In order to analyse the development of both the neural retina and RPE during early chick/human eye development, embryos were staged using the standard Hamburger-Hamilton system (Table 1)(Hamburger and Hamilton, 1951)/Carnegie stage system respectively. Whole eye globes were carefully dissected using watch-makers forceps. Prior to fixing in 4% PFA for 1 hour, incisions were made in the cornea of each eye using a sharp needle, in order to allow the fixative to properly permeate the tissue.

Hamburger-Hamilton Stage of Chick Development	Equivalent no. of days in embryonic chick development	Equivalent no. of days in human development	Equivalent Carnegie stage of human development
HH18	3 days	33 days	CS14
HH19	3-3.5 days	36 days	CS15
HH21	3.5 days	40 days	CS16
HH22	3.5-4 days	41 days	CS16
HH23	4 days	41.5 days	CS16
HH24	4.5 days	41.75 days	CS16
HH25	4.5-5 days	42 days	CS17
HH26	5 days	43 days	CS17
HH27	5-5.5 days	44 days	CS18
HH28	5.5-6 days	46 days	CS18
HH29	6-6.5 days	48 days	CS19
HH30	6.5-7 days	50 days	CS19

Table 1.
Table comparing the various stages of chicken and human development, including a comparision of the equivalent Hamburger-Hamilton, and Carnegie Stages, of development (Hill, 2010).

2.2 Isolation of embryonic human and chick RPE sheets:

In order to isolate RPE sheets, whole eye globes of the appropriate developmental age were removed from developing embryos using watch-makers forceps. The eyes were subsequently placed in PBS (Ca^{2+}/ Mg^{2+})- to aid the removal of adjacent tissues from the RPE. The retina, lens, cornea, and the majority of the extraocular mesenchyme were subsequently removed using fine forceps to leave an intact, pigmented RPE monolayer. It was important to

ensure that all retinal tissue had been removed from the RPE sheets through careful observation of RPE explants using a dissecting microscope.

In later chick experiments and all human experiments, RPE monolayers were purified of all contaminant tissue using gentle enzymatic treatment with dispase solution. Intact RPE globes were initially transferred to 0.03% dispase (Sigma) solution in Knock Out^{TM} DMEM basal medium (GIBCO) and allowed to incubate at room temperature for approximately 30 minutes. Following this period of incubation, tissues attached to the RPE monolayer were observed to have loosened, which subsequently allowed them to be removed more easily. The cornea, lens and extra-ocular mesenchyme were removed from the intact eye globe using the tip of a sharp needle, leaving a sheet of pure RPE and retina. The retina was then carefully removed from the RPE sheet, taking care not to leave any retinal contaminant. Once the RPE sheet was detached from the retina, pure RPE sheets were briefly washed in PBS and then immediately transferred to the appropriate culture medium. Post-dissection controls were fixed immediately following dissection and analysed for their purity, and the expression of retinal markers.

Critically, only the central most portion of the chick RPE monolayer was utilized for transdifferentiation experiments, given that cells in the CMZ region is known to undergo a different type of retinal regeneration in response to bFGF (Spence et al., 2007). Therefore, in order to remove any possibility of these cells being present in the RPE preparations, large regions at the periphery of the RPE sheet were removed using a sharp needle.

2.3 Transdifferentiation of RPE cells:

Several experiments employed the use of a standard culture system for transdifferentiation of the RPE towards a neuro-retinal phenotype.
RPE explants were analysed for the potential for transdifferentiation at several different stages of development, in a number of different culture media. The standard culture system for transdifferentiating RPE cells was very similar to that used is previous reports of the

phenomenon (Pittack et al., 1991, Sakami et al., 2008). RPE monolayers were cultured as intact explants in bacteriological dishes (Nunc), which prevented the attachment of the RPE monolayers to the base of the dish, therefore allowing the explants to remain in suspension. It was important to periodically agitate the dishes every 12 hours or so in order to ensure that the explants remained in suspension, and did not attach to the dish. Explants were cultured for a period of time in either HESC medium (80% KO DMEM + 20% KO serum, 1% non-essential amino acid solution, 1mM L-glutamine, 0.1mM β-mercaptoethanol), or a control medium previously shown to facilitate transdifferentiation, DMEM/F12 + 1% FBS (Pittack et al., 1991). Human cells were cultured for 10 days and chick cells were cultured for 7 days, after which they were fixed for analysis. Cells were incubated at 37°C in 5% CO_2 for the entire culture period. In order to initiate transdifferentiation of the RPE towards a neural retina phenotype, RPE explants were treated with 100ng/ml bFGF (Invitrogen), which has previously been shown to be sufficient for induction of transdifferentiation (Sakami et al., 2008, Pittack et al., 1991). bFGF was exogenously added to RPE explant cultures from the outset, and fresh doses were subsequently added to the culture medium every other day, in tandem with the replacing of half the culture medium with fresh medium at the same intervals.

2.4 Immunohistochemistry:

2.4.1 Sectioning of tissue

Whole eyes were fixed for 1 hour in 4% PFA the following day cells were then cryo-protected overnight in 30% sterile sucrose solution. Similarly, cultured RPE explants, as well as HESC-RPE cultures, were fixed for 1 hour in 4% PFA and subsequently cryo-protected in sterile, 30% sucrose solution overnight. Cryo-protected tissue was frozen in O.C.T. using a dry ice/alcohol bath. Tissue was the sectioned into 10 micron slices on a cryostat, and melted onto charged Superfrost^{TM} (Thermoscientific) slides.

Adherent RPE monolayers were similarly fixed for 1 hour in 4% PFA at 4°C and stored in ice-cold PBS solution until ready for labeling.

2.4.2 Antibody labeling of tissue sections.

All tissue was blocked in 5% normal donkey serum (NDS) in 0.3% Triton-X for 30 minutes at room temperature to prevent non-specific binding of antibodies. The blocking solution was removed and slides exposed to primary antibodies (listed in Table 2) in 1% NDS, 0.3% Triton-X overnight at room temperature. The primary antibody solution was discarded and the slides washed 3 times in 1 X PBS to remove any excess antibody. Tissue sections were exposed to 2% NDS, 0.3% Triton-X solution containing Rhodamine (TRITC)-, Fluorescein (FITC)- or Cy5TM-congugated AffiniPure Donkey secondary antibodies raised in mouse, rabbit or goat (Table 3) for 2 hours at room temperature. The secondary antibody solution was discarded, slides washed twice in 1 x PBS before tissue sections were exposed to 4'-6'Diamidino-2-phenylindole (DAPI) in 0.3% Triton-X for 1 minute at room temperature. The DAPI solution was discarded, slides were washed twice in 1 x PBS and three times in Tris-buffer before sections were mounted using vectorshield and a glass coverslip.

Slides were imaged on a Zeiss 700 confocal microscope and viewed in LSM browser. All immunohistochemical analysis was performed in parallel with both a positive labeling control, to ensure the activity of the antibody, and a no primary antibody negative control, which accounts for non-specific binding of the secondary antibody to the tissue.

Marker	Additional Comments	Raised in	Optimum Concentration	Source
Pax6	-	Rabbit	1:300	Covance
Sox2	-	Goat	1:200	Santa Cruz Biotechnology
HuD	-	Goat	1:300	Santa Cruz Biotechnology
Rhodopsin-4D2	-	Mouse	1:500	Millipore
Otx2	-	Rabbit	1:1000	Chemicon
Crx	-	Mouse	1:1000	Santa Cruz Biotechnology
Pmel17	Didn't work in chick	Mouse	1:100	DAKO
FGFR-1	-	Mouse	1:50	Invitrogen
alphaA-Crystallin	-	Rabbit	1:200	Santa Cruz Biotechnology
Nestin	-	Goat	1:200	Santa Cruz Biotechnology
Islet-1	-	Mouse	1:10	Developmental studies hybridoma bank
Chx10	Didn't work in chick	Goat	-	Santa Cruz Biotechnology
M/L-opsin	-	Rabbit	1:5000	Millipore
Mitf	Didn't work in chick	Mouse	1:300	ThermoScientific

Table 2
List of primary antibodies used in immunohistochemical analysis.

Name	Raised in	Optimal Concentration	Antigen	Source
TRITC-conjugated Anti-Rabbit	Donkey	1:100	IgG	Jackson Biosciences
FITC-conjugated Anti-Goat	Donkey	1:100	IgG	Jackson Biosciences
FITC-conjugated Anti-Mouse	Donkey	1:100	IgG	Jackson Biosciences
FITC-conjugated Anti-Rabbit	Donkey	1:100	IgG	Jackson Biosciences
TRITC-conjugated Anti-Mouse	Donkey	1:100	IgG	Jackson Biosciences
Cy5-conjugated Anti-Mouse	Donkey	1:100	IgG	Jackson Biosciences
Cy5-conjugated Anti-Goat	Donkey	1:100	IgG	Jackson Biosciences

Table 3
Table of secondary antibodies used in the immunohistochemical analysis of gene expression.

2.5 Image analysis using ImageJ software:

To quantify the levels of expression of RPE marker Pmel17, as well as the level of pigmentation in cultures of HESC-RPE and human fetal RPE cells, it was necessary to use an image analysis software, ImageJ. ImageJ is a software program which is able to convert single channel, coloured, nomarsky/fluorescent images to a 16-bit grayscale image. This involves creating an image where the level of pixel intensity is scored on a scale where regions of complete black = 0, and areas of complete white = 255. Therefore, it is possible to use these images to calculate an average pixel intensity value for each image, which corresponds to the average, relative pixel intensity for the image. See each chapter for specific methods applicable for each experiment.

2.6 Total RNA isolation:

In order to analyse the gene expression of RPE cells at the transcriptional level, it was necessary to isolate the total RNA from samples of HESC-RPE monolayers cultured on Matrigel for a period of 5 weeks in standard HESC-medium –bFGF, as well as transdifferentiated chick RPE treated +bFGF. The protocol for total RNA isolation was as follows:

1. Discard the culture medium and wash the cells 3 times in PBS to remove excess medium.
2. Aspirate the final PBS wash and add 500µl ice cold Tri-reagent to the culture dish and use the pipette tip to scrape the cells off the surface if the culture dish. Once the cells are removed from the surface of the tissue culture plastic, the suspension can then be transferred to a 1.5ml centrifuge tube. Once transferred, the cells were lysed with the aid of repeated pipetting in addition to being passed through a fine needle. This step was performed on ice to prevent degradation of RNA.
3. Once the cells are fully-lysed, the suspension is centrifuged at 12,000g for 10 minutes at 4°C to pellet the waste material. Following centrifugation, the supernatant contains RNA/DNA and protein. This is subsequently transferred to a fresh 1.5ml centrifuge tube and the pellet is discarded.
4. The samples were allowed to stand for 5 minutes at room temperature.
5. 100µl of cholorform was added in order to phase separate the RNA from both the DNA and protein in the sample. Following the addition of chloroform, samples were vigorously shaken for 15 seconds to mix, and then allowed to stand for a further 5 minutes at room temperature.

6. The sample was then centrifuged at 12,000g for 15 minutes at 4°C and the mixture will separate into 3 different phases:
    a. A red organic phase which contains protein.
    b. A white interphase which contains DNA.
    c. A colourless upper aqueous phase which contains RNA.
7. The upper aqueous phase containing the total RNA portion from the sample was then transferred to a fresh 1.5ml centrifuge tube, taking care not to disturb the other phases. 250µl of isopropanol was then added and the samples were left to stand for 5 minutes at room temperature in order for the RNA to precipitate as a white solid.
8. After precipitation, samples were centrifuged at 12,000g for 10 minutes at 4°C in order to pellet the precipitate.
9. Once the RNA pellet has formed, the supernatant was carefully removed and discarded, taking care not to disturb the pellet. The RNA was then washed by adding 1ml of 75% ethanol, and then vortexed.
10. In most cases, vortexing causes the pellet to float and therefore it is necessary to re-pellet the RNA by centrifuging the sample at 12,000g for 5 minutes at 4°C.
11. The supernatant was once again discarded and the pellet was left the air-dry for 5-10 minutes.
12. Once relatively dry (but not completely dried out) the pellet was re-suspended in 1 x RNASecure solution by pipetting at 55-60°C for 10-15 minutes.
13. This sample of total RNA can then be quatified using a nanodrop, used to produce cDNA, or stored at -80°C.

2.7 DNase treatment and reverse transcription of RNA to make cDNA:

It was subsequently necessary to produce cDNA form the total RNA sample, so that cloning reactions such as PCR could be performed. Firstly, in order to ensure that any DNA present in the final sample is produced from the total RNA, and not as a result of genomic or other contamination of the total RNA sample, it was necessary to treatment the total RNA with DNase I (Invitrogen) in order to remove any DNA which may be present in the initial sample. The protocol for this was as follows:

1. Mix the following components to a volume of 10µl, in a 500µl centrifuge tube for the DNase reaction
    a. 500ng-1µg of total RNA diluted in H_2O
    b. 1µl of 10X DNase buffer
    c. 1µl of DNase I enzyme solution
2. Incubate the reaction at room temperature for 15 minutes to allow to DNase reaction to take place.
3. Terminate the reaction by adding 1µl of (well mixed) 25mM EDTA to the sample and incubate at 65°C for 10 minutes.
4. Immediately chill the sample on ice.

Once the sample has been treated with DNase, it should be free of any DNA which was present in the intial sample, following RNA isolation. It is then possible to use this pure total RNA to produce cDNA using a reverse transcriptase reaction (Superscript-III, Invitrogen). The protocol for which is as follows:

On ice:-

1. Mix the following for each sample:
    a. 10µl of 2X reverse transcriptase reaction mix.

b. 2µl of reverse transcriptase enzyme mix.

c. 8µl DNase treated RNA.

2. Using a thermocycler, incubate the sample using the following program:

    a. 25°C for 10 minutes.

    b. 50°C for 30 minutes.

    c. 85°C for 5 minutes.

    d. Chill on ice

3. In order to digest the original total RNA template, 1µl of RNase H was added to the sample which was subsequently incubated at 37°C for 20 minutes.

The cDNA can now be stored at -20°C ready for use in experiments such as PCR.

2.8 Polymerase Chain Reaction (PCR):

PCR reactions were employed to attempt to clone genes of interest from cDNA. In order to control for DNA contaminants, in addition to cDNA made from samples of HESC-RPE, no reverse transcriptase controls (where the cDNA production reaction was performed without the addition of the reverse transcriptase enzyme) and no cDNA template controls were employed. It is important to run no reverse transcriptase controls to ensure that any product observed in the experimental sample has resulted from cloning of cDNA, and not any other DNA contaminant, for example, genomic DNA, which may have evaded degradation by the DNase treatment. Similarly, no template controls are important to ensure that any experimental products are not as a result of cloning of contaminant DNA products which may accidentally be present in PCR reagents. The protocol for standard PCR reactions using a Pfu DNA polymerase enzyme kit (Applied Biosystems) in this chapter is as follows:

1. Mix the following reagents:

a. 2.5µl 10x NH_4 to give a final concentration of 1x.

b. 1.5µl of 50mM $MgCl_2$ to give a final concentration of 3mM.

c. 1µl of dNTP mastermix to give a final concentration of 200µM.

d. 0.5µl of both forward and reverse primers (25µM) to give a final concentration of 500nM (See Table 4/5 for list of primers).

e. 0.25µl of Pfu polymerase to give a final concentration of 0.01-0.05U.

f. 0.5µl of template.

g. 18.75µl of H_20.

Once the reagents have been thoroughly mixed, the samples are incubated in a thermocycler using the following program:

Step	ºC	Time	
Denature	94	3 min	
Denature Anneal Extend	96 60 72	15 sec 30 sec 1 minute	35 cycles
Extend	72	4 min	

Samples were then analysed using 2% agarose gel electrophoresis.

Target	Forward	Reverse	Sequence (Forward)	Sequence (Reverse)
FGF-1R	1301+	1878-	ACTCTGGGGTTCTTCTGGTTCG	TTCATCACATTGTCCTCTGTCACC
Mannose-6-phosphate	1874+	2104-	ACCGTGGAGAGCCTGTTCTA	GTTTGGGTCATCGGAGAAGA

Table 4
A table of primer sequences used for PCR of human transcripts.

	Primer sequences		
Gene	**Forward**	**Reverse**	**Product length**
Rhodopsin	AGCCGGAGATCAACAACGAA	CTGGTGGAGACGGAGGAGGT	454bp
GAPDH	GACAGCCATTCCTCCACCTT	TCCAACAAAGGGTCCTGCTT	195bp

Table 5
A table of primer sequences used in the analysis of embryonic chick tissue.

2.9 Gel extraction and DNA sequencing:

In order to confirm that positive bands in the gel electrophoresis analysis were indeed the correct PCR products, it was necessary to isolate the cloned DNA and sequence it. Gel bands were cut out of gels using a fresh, sharp scalpel and the DNA product was purified using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

Purified products were then sequenced using a BigDye^{TM} sequencing kit (Life Technologies) using the following protocol:

1. Mix the following reagents for the sequencing reaction. Note there is one sequencing reaction for each individual primer, forward and reverse, for each PCR product.

a. Add 0.5µl of 1 primer.

b. Add 5.5µl of template (PCR product).

c. Add 4µl of BigDye sequencing mix.

d. Add 2µl of BigDye (BD buffer).

e. Add 8µl of water.

2. Once thoroughly mixed, the sequencing reaction was run on a thermocycler using the following program:

Step	ºC	Time	
Denature	95	3 min	
Denature	96	15 sec	35 cycles
Anneal	50	30 sec	
Extend	60	4 min	
Hold	10	-	

3. Once the sequencing reaction is finished, it is necessary to precipitate the DNA by addition of the following reagents:

0.5µl of 0.5M EDTA

2µl of 3M NaOAc pH5

50µl of Benzene free Ethanol

The samples were incubated with the above reagents at room temperature for 15 minutes.

4. Precipitated DNA was then centrifuged at 13,000 g for 30 minutes at 4°C to pellet the DNA.

5. Remove the supernatent.

6. Add 70µl of 75% ethanol to each pellet to wash it.

7. Centrifuge the samples at 13,000 g for 15 minutes at 4°C to re-pellet the DNA.

8. Remove the supernatent and air dry the pellet for 5-10 minutes until most of the ethanol has evaporated.

9. Immediately prior to loading into the sequencer, dissolve the pellets in 12µl of Hi Di formamide.

10. Denature the product by incubating at 95°C for 3 minutes and then place on ice.

11. Load onto the sequencer.

Sequences were analysed using the 4Peaks™ software and were aligned to expected sequences to confirm the identity of the product. Alternatively, sequences could be analysed using the BLAST program to reveal the identity of the gene from which the PCR product was cloned.

2.10 Production of HESC-RPE:

2.10.1 Isolation and culture of human embryonic stem cells

A human embryonic stem cell line derived from the inner cell mass of a 5 day old embryo, S*hef1*, was cultured using a standardized mouse embryonic fibroblast (MEF) co-culture technique (Vugler et al, 2008).

The MEF's were isolated from E13.5 embryos taken directly from pregnant mice. Pregnant mice were sacrificed using a schedule 1 method and their abdomens were sterilized using 70% ethanol to reduce chances of bacterial contamination. An incision was then made in the skin of the abdomen using surgical scissors to expose the uterine horns, which were subsequently removed and washed in 1 x PBS solution (w/o Ca^{2+}/Mg^{2+}). The embryos were isolated from the embryonic sacks removing the placenta and embryonic membranes from the embryos themselves. Embryos were then decapitated using a sharp scalpel, internal organs were removed and the bodies washed in 3 x PBS (w/o Ca^{2+}/Mg^{2+}) to remove any waste tissue/blood. The remaining embryo carcass' were then placed in a fresh dish, minced using a scalpel, and treated with trypsin (0.25% w/v) /EDTA (5mM) for 10-20 minutes at 37°C to dissociate cells from the embryo. After the incubation period, the enzymatic action of trypsin was quenched by adding an excess of DMEM/10% FBS/Pen/Strep (GIBCO). The embryo and solution was subsequently transferred to a large centrifuge tube and agitated vigorously in order to facilitate the removal of cells from the connective tissues of the embryo. The centrifuge tube was then left vertical at room temperature to allow the larger portions of connective tissue to settle under gravity. The remaining dissociated cell homogenate was

carefully transferred to a T75 tissue culture flask and fresh media added. Flasks were incubated at 37°C overnight to allow attachment of cells. The next day, the medium was replaced to remove the dead cells and other debris that did not attach to the surface of the flask overnight. MEFs were expanded to 90% confluency, passaged using trypsin/EDTA (GIBCO) and split at an appropriate ratio in DMEM/10% FBS/Pen/Strep.

Prior to use in embryonic stem cell culture, the MEFs must be mitotically inactivated to prevent sunsequent proliferation. To achieve this, confluent flasks of MEFs were passaged to give sub-confluent flasks containing proliferative fibroblasts. MEFs were used for HESC culture only up to passage 5, as it has been reported that MEF's at lower/higher passages are less effective in HESC culture (personal communication). MEFS were treated for 2-3 hours with mitomycin-C (10μg/ml) in DMEM/10% FBS at 37°C, the solution was then aspirated from the flasks and the cells were washed 3 times in 1x PBS to remove any excess solution. Cells were dissociated using trypsin (0.25%)/ EDTA (5mM) for 5 minutes at 37°C. DMEM/10% FBS medium was added to inactivate the enzymatic action of the trypsin, cells were pelleted by centrifugation at 200xg for 5, waste supernatant was removed by aspiration and the pellet resuspended in fresh medium. Cells were counted using a haemocytometer and then plated onto T25 tissue culture flasks at a cell density of 10,000 cells per cm^2 (a total of $2.5x10^5$ cells per flask).

Shef1 stem cell colonies were passaged onto inactivated MEF feeders and allowed to expand at 37°C + 5% CO_2 for a number of days. This was achieved by enzymatic dissociation, using a combination of sterilized glass beads and 0.04% collagenase IV (GIBCO) to gently dislodge the colonies from the surface of the flask, or through manual mechanical transfer of individual HESC colonies using a plastic pipette tip to dislodge stem cell colonies using a cell microscope. To preserve the pluripotency and proliferation HESCs are cultured in a HESC medium containing KnockOut™ DMEM (80%) + KnockOut™ serum replacement (20%) + 1% non-essential amino acid solution + 1mM L-glutamine + 0.1mM β-mercaptoethanol + 4ng/ml bFGF at 37°C + 5% CO_2 with daily replacement of media.

2.10.2 *Generation of HESC-RPE:*

HESC-RPE was generated through spontaneous differentiation of HESC colonies. HESC colonies were allowed to expand on inactivated MEFs with daily replacement of media until the colonies merged. At this point, normally after 7 days of culture, bFGF was removed from the HESC media and spent medium replaced with HESC-bFGF every other day for a number of weeks. The first pigmented HESC-RPE foci were usually visible at around 3 weeks post-removal of bFGF from the culture medium. The size and number of HESC-RPE foci within super-confluent HESC flasks varied, but increased over time in culture.

Pigmented HESC-RPE foci were mechanically isolated from the heterogenous differentiated stem cell population under a dissecting microscope using a cresent blade. Pigmented foci were transferred to collagenase IV solution (0.04%) at room temperature for 30 minutes in order to facilitate the removal of contaminating tissue. Following collagenase treatment, foci were washed in 1 x PBS (w/o Ca^{2+}/Mg^{2+}), any remaining contaminant tissue was removed using a fine needle and the isolated HESC-RPE foci pelleted by centrifugation at 500xg for 5 minutes.

2.11 Statistical analysis:

As a consequence of there being a small number of samples per group size, a RANOVA was performed. This is a preferred test when compared with both Kruskl-Wallis, or a standard ANOVA. The RANOVA allows multiple iterations of the data to produce randomised datasets, to power the group sizes. As with a standard ANOVA, the output is an F value with known degrees of freedom, thus allowing a probability to be calculated.

Chapter 3 –

Characterisation & development of the chicken model of RPE to neural retina transdifferentiation.

3.0 Introduction

It is currently unclear as to whether or not human RPE cells are able to undergo transdifferentiation of their RPE towards a neuroretinal phenotype. It is difficult to investigate this phenomenon in detail in human tissue for a number of reasons. Firstly, the difficulty in obtaining sufficient quantities of human fetal RPE tissue limits the number of experiments that can be performed during any investigation. In addition to this, it is very difficult to obtain human RPE tissue at a comparable developmental stage to that reported to transdifferentiate in response to bFGF.

It is therefore a sensible course of action to employ one of the animal models in order to properly characterize the phenomenon of transdifferentiation in the context of this investigation. This raises the question of which animal model is the most appropriate for the comparison with possible human RPE transdifferentiation, both scientifically and practically. Despite all models of transdifferentiation having been referred to as 'regeneration' of the retina, there are actually distinct differences between some of the models. It appears that each model exhibits similar molecular controls which govern the onset, and maintenance of RPE transdifferentiation, however, the key differences suggest that the regenerative phenomena are not necessarily the same event. The semantics of the events surrounding transdifferentiation can at times be confusing given that there is considerable cross-over in the use of terms like 'transdifferentiation' and 'regeneration' in the literature. The *urodeles* (newt) model of transdifferentiation is somewhat unique in that it appears to involve a pure, regenerative event. This is characteristic of a species that can regenerate a number of tissues, including its limbs, lens and retina (Susaki and Chiba, 2007, Chiba et al., 2006b, Ikegami et al., 2002, Grigoryan, 1993, Araki, 2007, Mitashov, 1997, Mitashov, 1996, Mitashov et al., 1995, Mitsuda et al., 2005, Kaneko and Chiba, 2008, Eguchi, 1988, Brockes and Kumar, 2005, Brockes, 1997). The newt RPE does undergo transdifferentiation to produce cells with a neuroretinal phenotype, however, in contrast to other models of RPE transdifferentiation, the regenerated retina which results from this event has the same laminar structure as the native retina, with

the ganglion cell layer on the inner layer of the retina, and the photoreceptor cells immediately adjacent to the RPE monolayer. In addition to this, the regenerating retina is also able to regenerate the RPE from which it has transdifferentiated, leaving the newt with a fully-functional retina resembling the native retina (Chiba et al., 2006a). Crucially, the newt has the ability to undertake this process throughout the life of the animal, which allows it to repair the retina following damage or removal. Therefore, in this particular instance, the RPE could be said to act as a permanent adult stem cell which can be activated whenever the host has incurred damage.

In contrast, transdifferentiation of the RPE towards a neuroretinal phenotype in the rat, mouse and chicken is only able to occur at the very earliest of stages of embryonic development, with capacity becoming restricted soon after the differentiation of the RPE (Zhao et al., 1995, Sakami et al., 2008, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Pittack et al., 1997, Pittack et al., 1991). In addition, none of these species are known to have the specialized regenerative capacity like that of the newt. Despite this, the type of transdifferentiation of the RPE in these species has also been referred to as 'regeneration' of the retina. However, this gives a somewhat false impression that the resulting neuroepithelium is able to become a fully-functional, properly orientated and laminar retina. In reality, the neuroepithelium which results from transdifferentiation of the RPE in these species, despite its expression of retinal markers in the expected laminar fashion, appear to be inverted when compared to the native retina. Ganglion cell markers are found on the outer surface of the neuroepithelium, and photoreceptor markers on the inner surface (Opas and Dziak, 1994a, Sakami et al., 2008, Coulombre, 1981, Pittack et al., 1997, Pittack et al., 1991, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993, Reh et al., 1991). The inverse nature of the novel retina is thought to reflect the developmental ancestral lineage of the immature RPE from which it transdifferentiates. The RPE differentiates from the multi-potent monolayer of cells in the outer-layer of the optic cup, following invagination of the optic vesicle (Fuhrmann et al., 2000b, Muller et al., 2007, Nguyen and Arnheiter, 2000, Martinez-Morales et al., 2004, Martinez-Morales et al., 2001, Martinez-Morales et al., 2003).

These cells of the presumptive RPE are known to have an inverse polarity with respect to the presumptive neural retina, as a result of the invagination. It is therefore reasonable to assume that these cells should maintain this inverse-polarity following transdifferentiation into a novel neural retina. In addition, following transdifferentiation into neural retina, the host RPE is not regenerated like that of the regenerating newt, but remains absent. Both of these factors mean that the novel retina produced as a result of the RPE transdifferentiation would not be able to function properly, given that the presence of RPE cells and polarity of neural retina are crucial to the proper function of the overall retina. It is therefore somewhat inappropriate to refer to this model of RPE transdifferentiation as 'regeneration' of the retina, given that a functional retina cannot be re-produced. In reality, the characteristics of this model of transdifferentiation suggest that rather than a regenerative event taking place, the ability to transdifferentiate reflects to multi-potent nature of the cells of the outer optic cup, which subsequently differentiate into the RPE monolayer.

The fact that the RPE of these species can only transdifferentiate at embryonic stages might suggest that the RPE actually retain the multi-potent, stem cell-like properties of their developmental ancestors, even after they have already differentiated enough to already resemble RPE cells. This multi-potent capacity in response to growth factors could then be gradually restricted through molecular controls being imposed upon the putative RPE cells as they mature in development. This seems especially plausible given that the RPE cells are often most easily identified by their intense pigmentation and cobblestone morphology, and the capacity for RPE transdifferentiation in response to bFGF is reported to be lost soon after this phenotype develops.

The fact that the avian/mammalian model of RPE transdifferentiation appear to be conserved across a number of species, and that it appears as if this type of RPE transdifferentiation largely reflects the heavily conserved gross development of the optic cup, including immature RPE and neural retina, would imply that if human RPE cells are also able to undergo transdifferentiation, they are most-likely to behave in a similar manner and display this feature of development. Therefore, in order to investigate a model of transdifferentiation,

it is reasonable to assume that one of the avian/mammalian models rather than an amphibian model would be most appropriate. This is particularly true given that the practical aspects of working with amphibian can be difficult given the restricted number of biological resources, such as genome sequences and antibodies, are available. Ideally, a mammalian model of transdifferentiation would be most appropriate for comparison with human RPE cells given that the species are more-likely to behave in a similar manner, owing to their close phylogenetic relationship. However, there is limited literature available which describes the phenomenon of RPE to neuro-retinal transdifferentiation in mammals. This is not the case with the chicken model. The avian model is well established for developmental studies given the relatively short period of gestation *in ovo,* the ease of access to embryos for isolation of tissues and transplantation studies where required, and the relative abundance of biological resources available for investigation, including fully-sequenced genome sequences, accurate staging references, antibodies, vectors etc. It is also of benefit that many embryos can be obtained and maintained very easily, particularly in comparison to the high maintenance husbandry involved when using rodent models.

3.1 Characterisation of retinal markers in the developing chicken retina:

3.1.1 Introduction:

The capacity for transdifferentiation of embryonic chicken RPE in response to bFGF is reported to be lost after the HH24 stage. (Park and Hollenberg, 1989, Pittack et al., 1997, Pittack et al., 1991, Sakami et al., 2008). If this restriction is also apparent in developing human RPE cells, it will be important to understand the mechanisms for this loss in potential for transdifferentiation. The candidates responsible for the limitation of the capacity for transdifferentiation remain unknown, however, if they be identified, it may be possible to reverse these changes and regain the capacity for transdifferentiation in response to

exogenous treatment with bFGF. Therefore, a developmental study spanning stages from early specification of the RPE prior to pigmentation, through to later developmental stages where the RPE appears well formed and is reported to have lost the potential for transdifferentiation, was undertaken. This investigation analysed the expression of several markers that have been implicated in the process of transdifferentiation, as well as the development of both the retina and the RPE itself. It is hoped that this will shed light on the reason for the reported loss in potential for transdifferentiation in response to bFGF. This also has the advantage of giving a reference point for the comparison of transdifferentiated retina with that of the native retina. In addition, it also allows the characterisation of a number of antibodies in chicken tissue, which is important for on-going studies in the chicken model of RPE transdifferentiation.

3.1.2 Materials & Methods:

Chicken eyes of differing developmental stage were isolated from Hamburger-Hamilton stage 18 (HH18) and stage 27 (HH27) as described in chapter 2. These eyes were subsequently analysed for the expression of a number of retinal markers using the antibodies described in chapter 2.

3.1.3 Results:

A summary of the expression of retinal markers in developing chick eye can be found in table 3.1.

3.1.3.1 HuD:

Amacrine and ganglion cell marker, HuD, expression was absent from the RPE at all developmental stages analysed (Figs. 3.1, 3.2), which is consistent with a marker usually associated with a neuroretinal phenotype. No expression of HuD was observed in the retina at CS18, the earliest stage analysed in this investigation (Fig. 3.1i A, B). HuD first appeared to be expressed in the retina at stage HH19, where a very small number of cells in the central retina began to weakly express the protein within their cytoplasm (Fig. 3.1i D, E). These cells were generally localized to the vitreal surface of the neuroepithelium, however, some more weakly labeled cells were observed to lie in the middle of the layer. By the time the RPE has pigmented at HH22 (Fig. 3.1i I), more neuroepithelial cells were labeled for HuD expression, once again with the majority of these cells being localized to the vitreal surface of the retina (Fig. 3.1i G, H), and these cells appeared to express HuD at a higher level than the very few weakly labeled cells possibly located within the middle of the neuroepithelium. More HuD positive cells were observed by HH23, with the majority of cells lining the vitreal surface of the retina observed to express HuD in the central retina, in addition to a greater number of sporadic HuD positive cells within the neuroblastic layer (Fig. 3.1i J, K). The level of expression of HuD in the vitreally localized cells increased by HH24 (Fig. 3.1ii M, N) and HH25 (Fig. 3.1ii P, Q), with a gradual increase in number of cells, as well as the expression level of the transcription factor observed in the middle of the neuroblastic layer (Fig. 3.1ii N, Q). By HH26, the majority of HuD positive cells have become restricted to the distal, developing ganglion cell layer of the retina at the vitreal surface (Fig. 3.1ii S, T). Some HuD positive cells were observed within the middle of the layer, however it appeared as though there were fewer of these cells than at HH25 (Fig. 3.1ii Q). HuD expression was completely localized to the developing ganglion cell layer by HH27, with no observable cells being labeled within the middle of the neuroepithelium in the central-most portion of the retina (Fig. 3.1ii V, W).

		RETINA		RPE	
Marker	Developmental stage	Central	CMZ	Central	CMZ
HuD	HH18	-	-	-	-
	HH19	+	-	-	-
	HH22	+	-	-	-
	HH23	+	-	-	-
	HH24	+	-	-	-
	HH25	+	-	-	-
	HH26	+	-	-	-
	HH27	+	-	-	-
Islet1	HH18	+	-	-	-
	HH19	NA	NA	NA	NA
	HH22	+	-	-	-
	HH23	+	-	-	-
	HH24	+	-	-	-
	HH25	+	-	-	-
	HH26	+	-	-	-
	HH27	+	-	-	-
FGF-R1	HH18	+	NA	+	NA
	HH19	+	NA	+	NA
	HH22	+	NA	+	NA
	HH23	+	NA	+	NA
	HH24	+	NA	+	NA
	HH25	+	NA	+	NA
	HH26	+	NA	+	NA
	HH27	+	NA	+	NA
AACrystallin	HH18	+	+	-	-
	HH19	+	+	-	-
	HH22	+	+	-	-
	HH23	+	+	-	-
	HH24	+	+	-	-
	HH25	+	+	-	-
	HH26	+	+	-	-
	HH27	+	+	-	-
Nestin	HH18	-	-	-	-
	HH19	-	-	-	-
	HH22	+	-	-	-
	HH23	+	-	-	-
	HH24	+	+	-	-
	HH25	+	+	-	-
	HH26	+	+	-	-
	HH27	+	+	-	-
Pax6	HH18	+	NA	+	NA
	HH19	NA	NA	NA	NA
	HH22	+	NA	-	NA
	HH23	+	NA	-	NA
	HH24	+	NA	-	NA
	HH25	+	NA	-	NA
	HH26	+	NA	-	NA
	HH27	+	NA	-	NA
Sox2	HH18	+	NA	-	NA
	HH19	NA	NA	NA	NA
	HH22	+	NA	-	NA
	HH23	+	NA	-	NA
	HH24	+	NA	-	NA
	HH25	+	NA	-	NA
	HH26	+	NA	-	NA
	HH27	+	NA	-	NA

Table 3.1
A summary of retinal marker expression during chick retinal development.
Positive labelling (+), Negative labelling (-), Not applicable (NA).

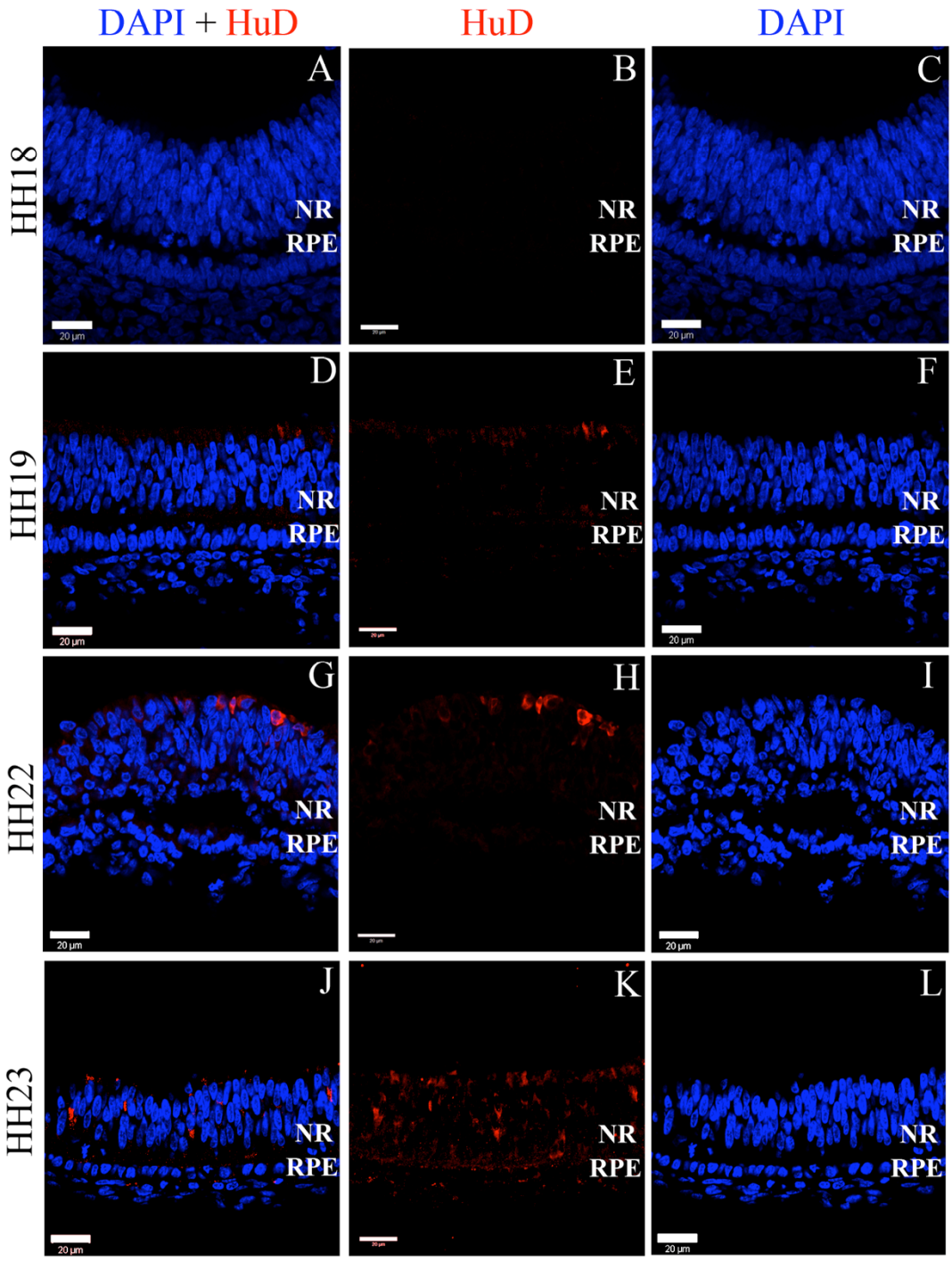


Fig. 3.1i
Developmental study of HuD expression in chick retinal development (early stages) in central retina.
HuD (red) is absent from HH18 central retina (B), as well as the RPE at all stages HH18 (B), HH19 (E), HH22 (H) and HH23 (K). HuD is first weakly expressed in very few cells at HH19 (E), and subsequently in many more cells by HH22 (H). These cells are generally localised to the vitreal surface of the neuroepithelium, in the presumptive ganglion cell layer as expected. This pattern is consitent at HH23 (K), but at this stage some cells are also observed to express HuD in the middle of the retina, possibly labeling amacrine cells, or migrating ganglion cells (K). However, most cells at HH23 reside at the vitreal surface in the developing ganglino cell layer (K). Merge (A, D, G, J), HuD (B, E, H, K), DAPI (Blue) (C, F, I, L). Fluoresence digitally enhanced. Scale bars: 20uM.

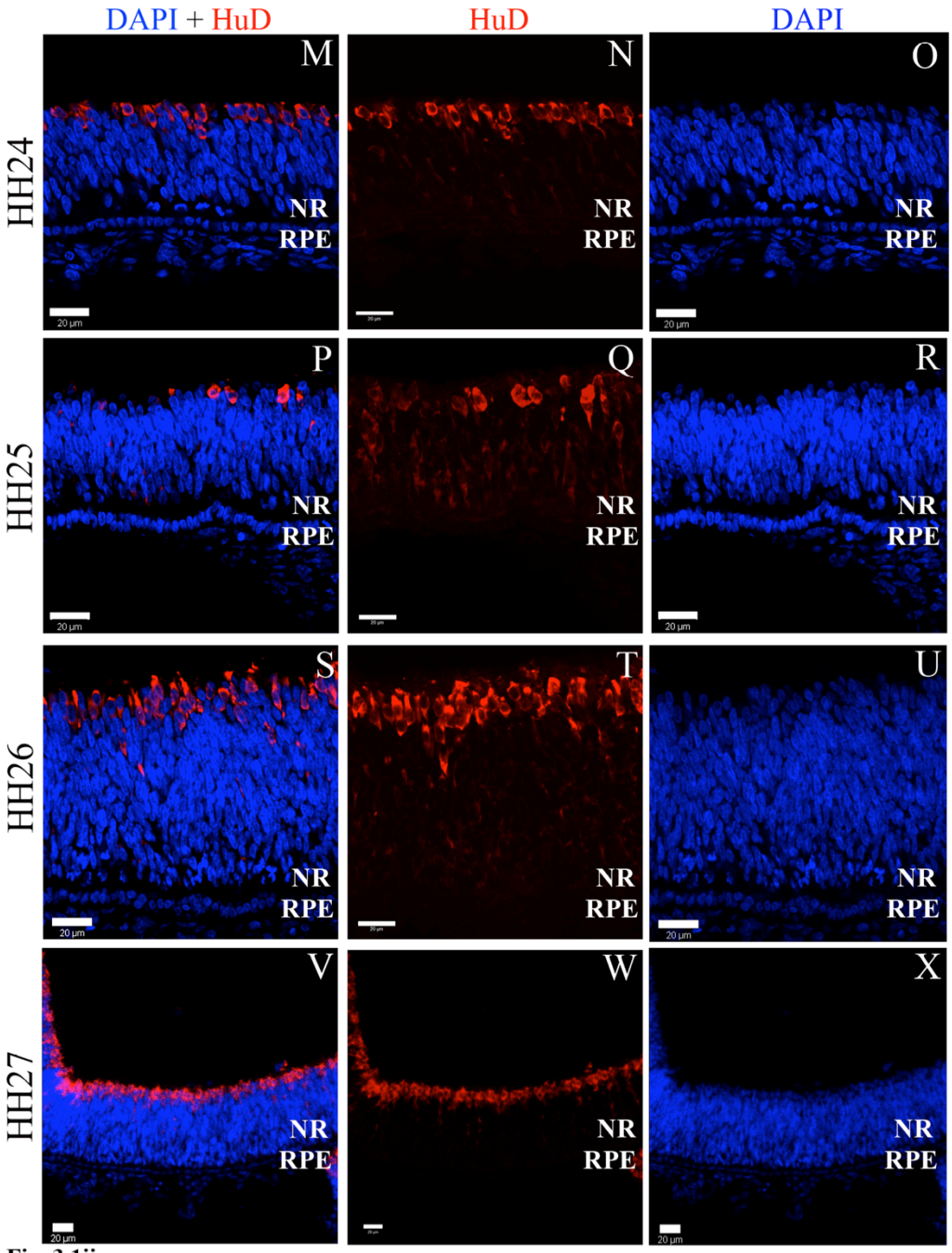


Fig. 3.1ii

Developmental study of HuD expression in chick retinal development (later stages) in central retina.

HuD (red) expression was negative from the RPE at all stages of development: HH24-HH27 (N, Q, T, W). HuD was largely localised to the cytoplasm of cells at the vitreal side if the retina, where developing ganglion cells are located at HH24 (N), HH25 (Q), HH26 (T), and HH27 (W). This region exhibited the highest level of expression of HuD at all stages investigated (N, Q, T, W). However, some expression was also observed in the middle of the neuroepithelium at all stages HH24-HH27 (N, Q, T, W), however, this expression was weaker than that at the vitreal surface. Additionally, expression of HuD in the middle of the retina became more restricted to the vitreal surface with progression of development until HH27, where the vast majority of cells expressing HuD were in the presumtpive ganglion cell layer (W). Merge (M, P, S, V), HuD (N, Q, T, W), DAPI (Blue)(Q, R, U, X). Fluoresence digitally enhanced. Scale bars: 20uM.

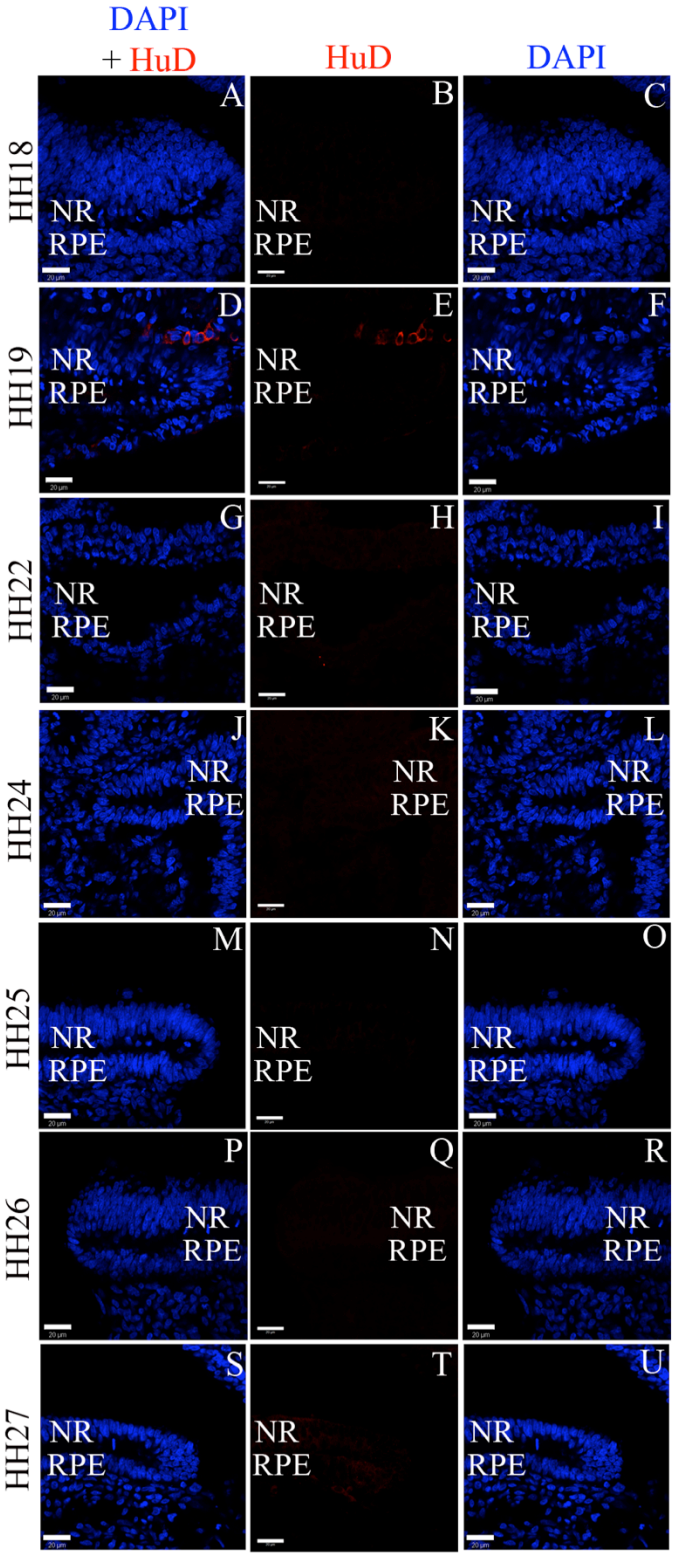


Fig. 3.2i

Developmental study of HuD expression in peripheral chick retinal development.

No HuD expression (Red) was observed in the peripehral RPE (RPE) at any developmental stage between HH18 and HH27 (B, E, H, K, N, Q, T). Similarly, HuD expression was absent from the non-pigmented neuroepithelium (NR) in this region between HH18 and HH27 (B, E, H, K, N, Q, T). Merge (A, D, G, J, M, P, S), HuD (B, E, H, K, N, Q, T), DAPI (Blue)(C, F, I, L, O, R, U). Scale bars: 20uM.

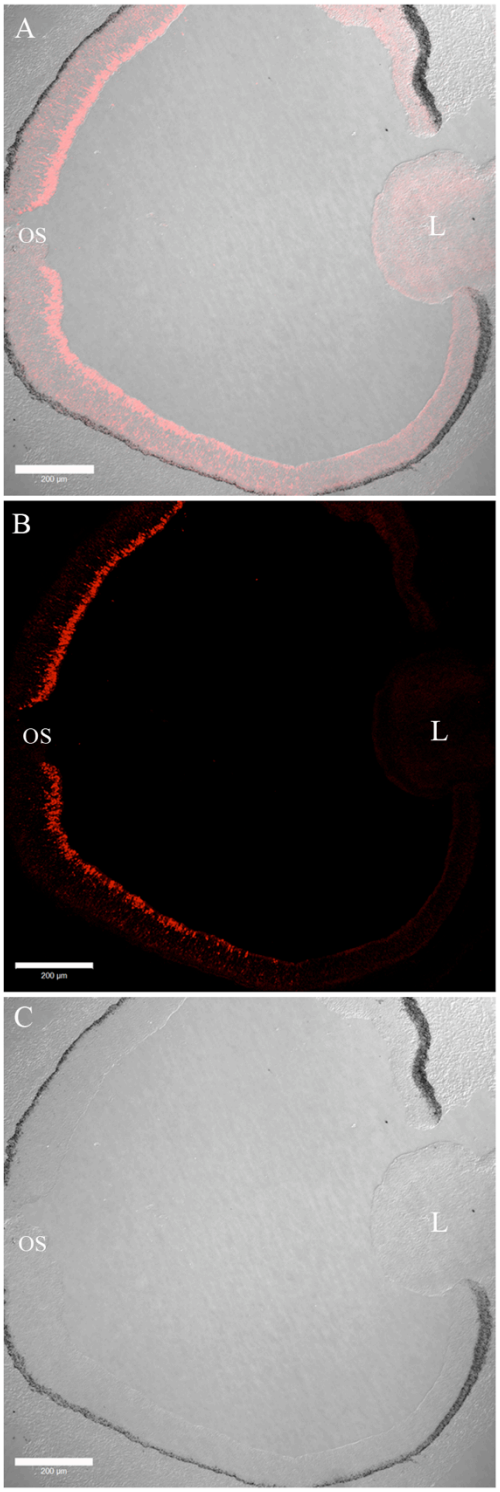


Fig. 3.2ii
Expression of HuD in the chick eye stage HH27.
The expression of HuD at HH27 was largely confined to the presumptive ganglion cell layer at the vitreal surface (B). No expression of HuD was observed in the RPE pigmented RPE as expected (A, B). Expression of HuD was highest in the central portion of the retina at the back of the eye adjacent to the optic stalk (OS), and the level of expression of HuD was observed to decrease in a gradient from this central region towards the periperhy of the retina, where no cells in the retina were obserevd to express HuD (B). Merge (A), HuD (B), Nomarsky (C). Lens (L). Fluoresence digitally enhanced. Scale bars: 200uM.

The expression of the HuD transcription factor was found to be highest in the central retina, adjacent to the optic stalk (Fig. 3.2iiA-C) and then gradually became reduced in a central to peripheral gradient, where no cells in the far peripheral ciliary marginal zone (CMZ) were observed to express the protein at any stage (Fig. 3.2iA-U). Interestingly, despite the fact that no HuD positive cells were observed in the middle of the neuroepithelium, in the central most portion of the retina (Fig. 3.1ii V, W), some cells in the middle of the neuroepithelium did express the protein in more peripheral regions (Fig. 3.2iiB).

3.1.3.2 Islet1:

Similarly, Islet-1 is also used as a marker for ganglion cell differentiation. As expected, the expression of this marker was absent from both non-pigmented RPE at early stages of development (Fig. 3.3iA-D), as well as in pigmented RPE at later stages (Fig. 3.3iE-H, 3.3iiA-H). Islet-1 was expressed in the nuclei of cells at the vitreal surface of the developing neural retina at every stage investigated, as early as HH18 (Fig. 3.3i). This contrasts with HuD expression, another marker of early amacrine and ganglion cell differentiation, which isn't expressed until slightly later in the development of the retina at HH19 (Fig. 3.1i). The number of cells expressing Islet-1 in the presumptive ganglion cell layer was generally observed to be somewhat variable at different stages of development, with perhaps a general increase in the number of cells with a progression of development (Fig. 3.3i, 3.3ii). New cells appeared to differentiate in a central to peripheral gradient with an observed central to peripheral expansion in the Islet-1 positive region with a progression of development (data not shown). No cells in the CMZ region were observed to express Islet-1 at any of the developmental stages analysed (Fig. 3.3iii B, D, F, H, J, I, N), and its expression appeared to be localized to the proximal retina over this developmental period, with a gradual decline in expression from the central retina (Fig. 3.4 yellow arrows) to the more peripheral retina, where their appeared to be a boundary at which Islet-1 expression rapidly becomes down-regulated (Fig. 3.4 red arrow).

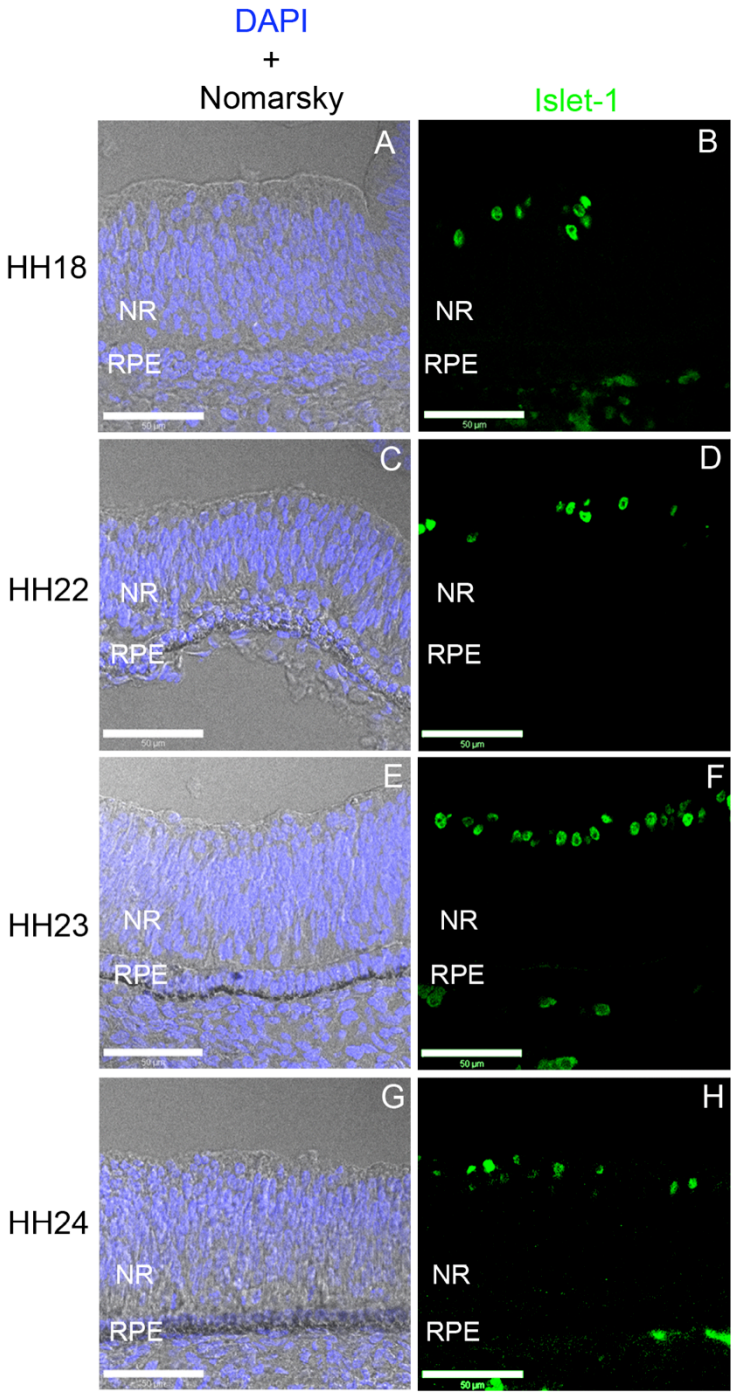


Fig. 3.3i

Developmental study of Islet-1 expression in central chick retinal development. Islet-1 (green) expression is localized to the nuclei of cells in the presumptive ganglion cell layer of the central retina at HH18 (B), HH22 (D), HH23 (F), HH24 (H). This corresponds with the birth of most ganglion cells in the retina and therefore suggests Islet-1 is a good marker of ganglion cell fate. The number of retinl cells expressing Islet-1 was observed to increase from HH18-HH24 (B, D, F, H). No expression was observed in the RPE or periperhal retina at any stage (B, D, F, H). DAPI (Blue). DAPI + Nomarsky (A, C, E, G), Islet-1 (B, D, F, H). Fluoresence digitally enhanced. Scale bars: 50uM.

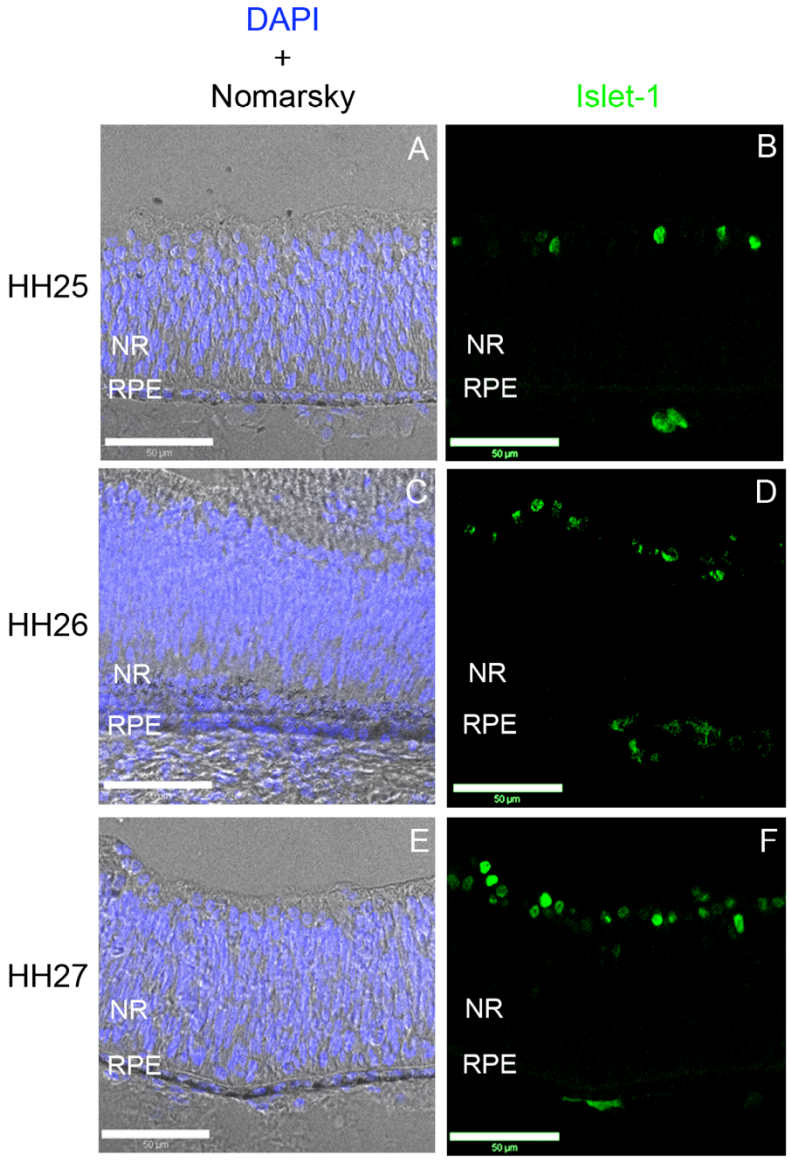


Fig. 3.3ii
Expression of Islet-1 in the developing central chick retina.
Islet-1 (green) was not observed in the pigmented RPE cells at any stage investigated between HH25-HH27 (B, D, F). However, Islet-1 expression was observed in the pre-sumptive ganglion cells at the vitreal surface of the neural retina (NR) at HH25 (B), HH26 (D), and HH27 (F). The apparent specificity of Islet-1 to the developing ganglion cell layer indicates that this marker is a good marker of ganglion cell fate. DAPI (Blue), DAPI + Nomarsky (A, C, E), Islet-1 (B, D, F). Fluoresence digitally enhanced. Scale bars: 20uM.

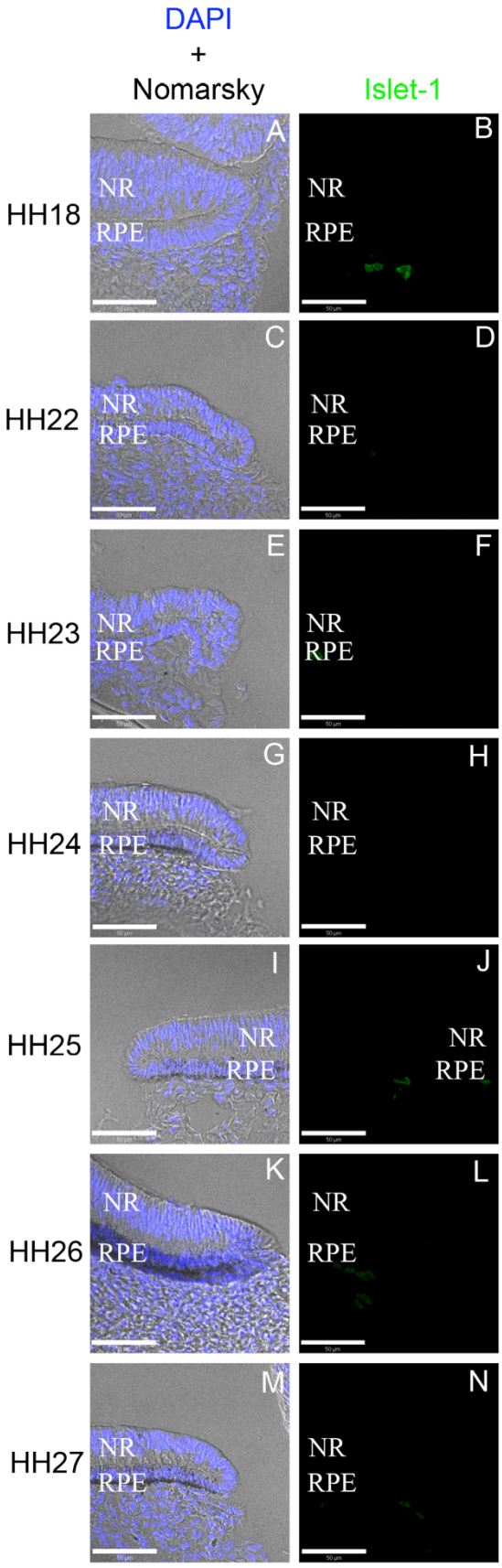


Fig. 3.3iii
Expression of Islet-1 in the peripheral chick retina.
Islet-1 (green) expression was absent from both pigmented RPE cells, and non-pigmented retinal cells (NR) at all stages investigated between HH18-HH22 (B, D, F, H, J, L, N). This suggests that no ganglion cells are present in this region of the retina, at least at these stages of development. DAPI (Blue). DAPI + Nomarsky (A, C, E, G, I, K, M), Islet-1 (B, D, F, H, J, L, N). Scale bars: 50uM.

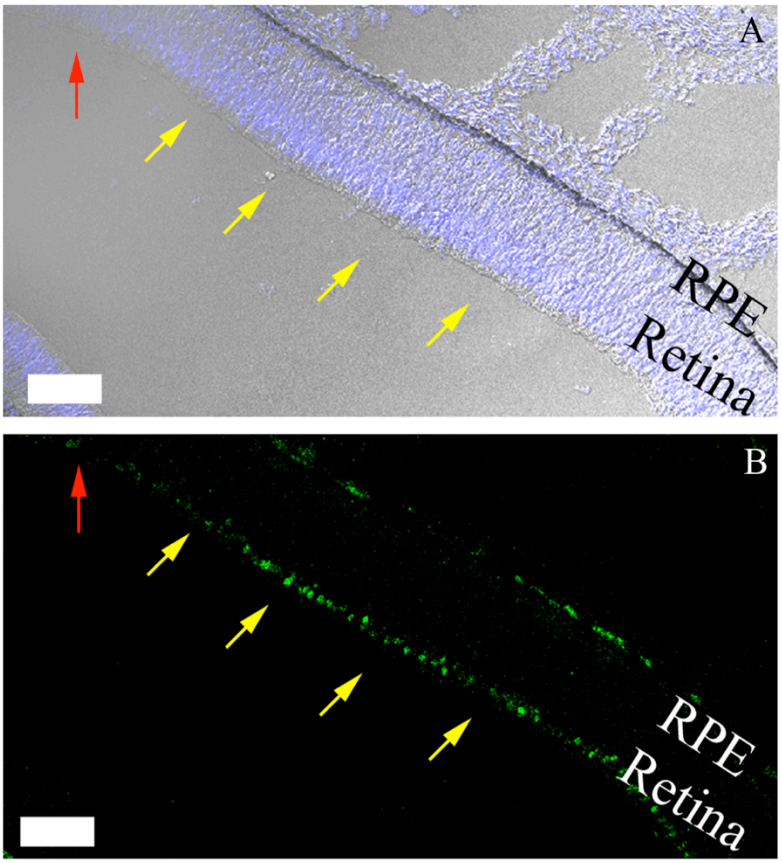


Fig. 3.4
Low magnification image of Islet-1 expression at HH27.
No expression of Islet-1 (green) is observed in the RPE (B) but is confined to the central retina at this stage (B yellow arrows). The signal apparent in the region of the RPE does does localise to the RPE itself, but instead is the background signal from cells beneath the RPE (B). Islet-1 expression is gradually reduced in a gradient from the central retina to peripheral retina, where Islet-1 expression is negative (B, red arrow). (A) Nomarsky + DAPI image showing retinal structure. Fluoresence digitally enhanced. Scale bars: 50uM.

3.1.3.3 Chx10:

The Chx10 antibody was unexpectedly observed to label an antigen in both the RPE and neural retina at all stages of development investigated (Fig. 3.5i B, D, F, H, J; 3.5ii B, D, F). This antigen did not appear to be localized to the nucleus but instead appeared to be largely localized to the cell membranes of both RPE and retinal cells (Fig. 3.5i B, D, F, H, J; 3.5ii B, D, F). In the RPE, Chx10 labeling was observed to be restricted to the apical side of the monolayer, adjacent to the developing neural retina, at all stages investigated (Fig. 3.5i B, D, F, H, J; 3.5ii B, D, F). Chx10 labeling in the neural retina was observed across the entire neuroepithelium, however, the most intense labeling occurred in the presumptive outer nuclear layer, immediately adjacent to the RPE monolayer at all stages (Fig. 3.5i B, D, F, H, J; 3.5ii B, D, F). Cells in the middle and at the vitreal surface of the neural retina exhibited less intense labeling than the outer region (Fig. 3.5i B, D, F, H, J; 3.5ii B, D, F).

3.1.3.4 FGF-R1:

The fibroblast growth factor receptor, FGF-R1, was observed to be robustly expressed throughout the neural retina and RPE at all stages investigated (Fig. 3.5i A, C, E, G, I; 3.5ii A, C, E). The fluorescence signal was localized to the cell membrane of all cells which is concurrent with the profile expected for a receptor protein (Fig. 3.5i A, C, E, G, I; 3.5ii A, C, E). The level of expression of the receptor appeared to be relatively consistent across all stages of development investigated, with comparable levels of expression observed in both the RPE and adjacent neural retina (Fig. 3.5i A, C, E, G, I; 3.5ii A, C, E). FGF-R1 was found to be expressed throughout the cell membrane of both neural retinal cells and RPE, both at the baso-lateral surface, and apical surface, unlike labeling observed for Chx10 (Fig. 3.5i A, C, E, G, I; 3.5ii A, C, E).

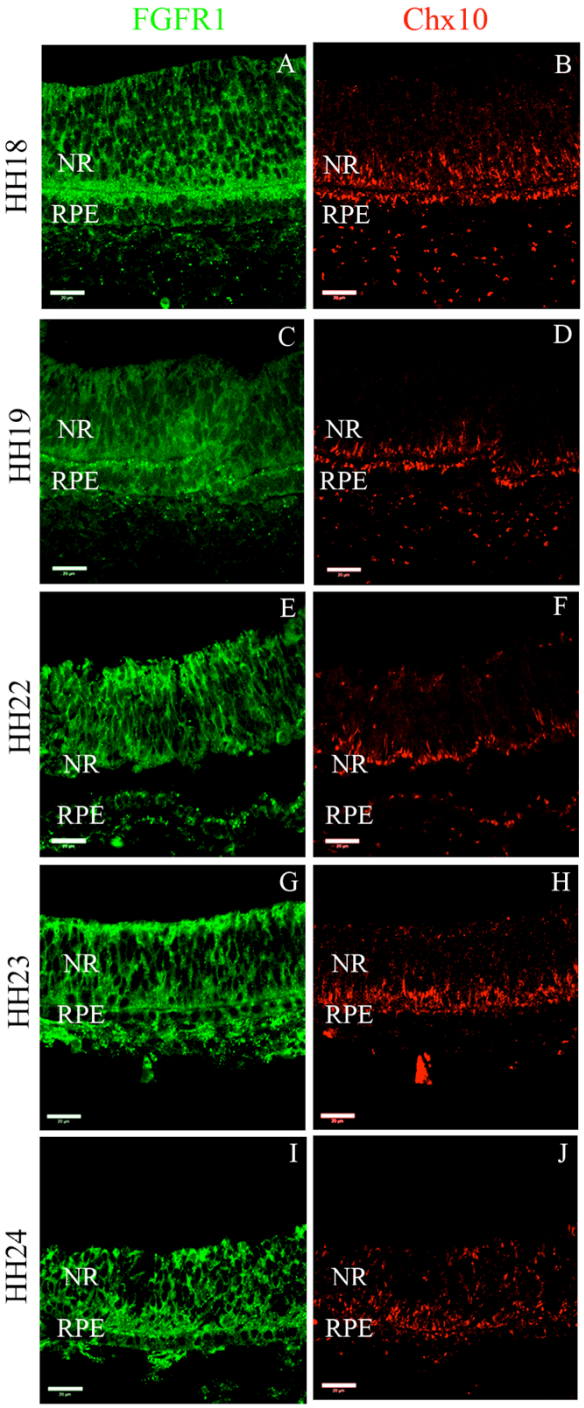


Fig. 3.5i
Developmental study of FGF-R1 and Chx10 expression in central chicken retina.
FGF-R1 (green) expression was robustly expressed in the cell membrane of both RPE and retinal cells at all stages of development investigated HH18-HH24 (A, C, E, G, I). This is consistent with previous reports of FGF signalling being important for retinal development. FGF-R1 expression in the RPE supports the idea that bFGF is able to signal to the RPE at these stages, for example, for initiation of transdifferentiation. Chx10 (red) also labeled the retina and RPE at all stages, however, the expression pattern was unexpectedly non-nuclear, and was localised to the apical surface of RPE, as well as the basal layer of the neuroepithelium (B, D, F, H). This would suggest that non-specific binding of the antibody may have taken place. Fluoresence digitally enhanced. Neural retina (NR). Scale bars: 20uM.

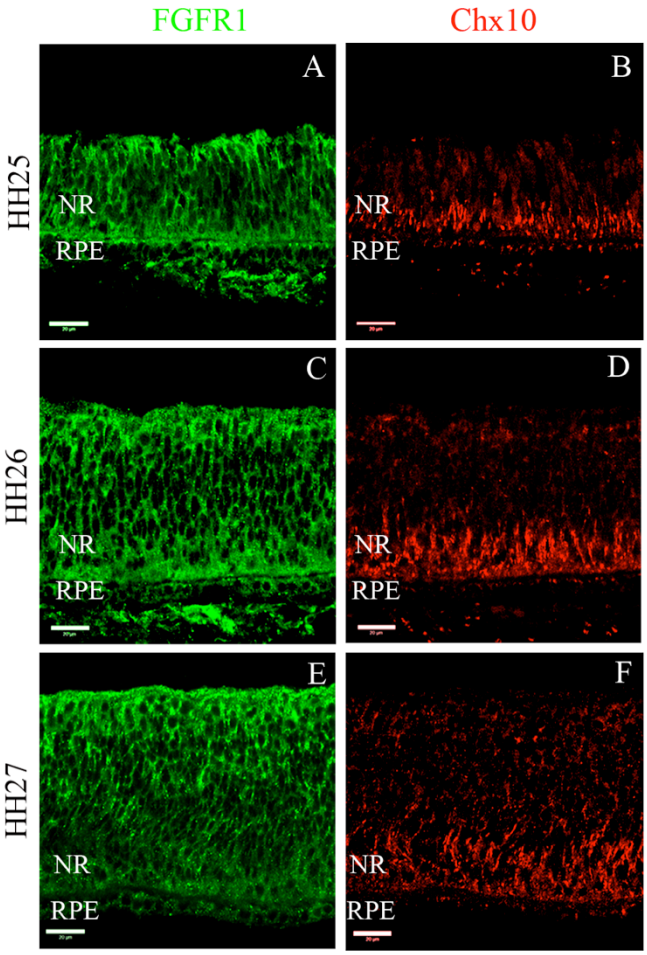


Fig. 3.5ii
Expression of FGF-R1 and Chx10 in the developing central chick retina.
FGF-R1 (green) is robustly expressed thoughout the neural retina (NR) and RPE (RPE) at stages HH25 (A), HH26 (C), HH27 (E). Expression is localised to the cell membranes which is consistent with the expression pattern expected for a membrane-bound receptor. RPE cells post-HH25 consistently express FGF-R1 which suggests that a loss in the expression of the receptor is not the cause of a loss in the potential for transdifferentiation. Chx10 (red) expression was localised to the cytoplasm of both neural retina (NR) and RPE (RPE) cells at these stages, instead of the nucleus as expected (B, D, F). This would suggest that the Chx10 antibody has undergone non-specific binding to an unknown antigen, and therefore, is unsuitable for analysis of Chx10 expression in development. Fluoresence digitally enhanced. Scale bars: 20uM.

3.1.3.5 αA-crystallin:

The major structural protein from which lens tissue is composed, αA-crystallin, was absent from the RPE at all stages of development investigated, both centrally (Fig. 3.6iB, F, J, N; 3.6iiB. F. J. N), and peripherally in the CMZ (Fig. 3.7iB, F, J, N; 3.7iiB. F. J. N). However, surprisingly, in addition to robustly labeling the developing lens, as would be expected (Fig. 3.7iiF, J, D, N), some weak labeling for the protein was detected within cells of the neural retina in central regions (Fig. 3.6iB, F, J, N; 3.6iiB. F. J. N), however, the signal which appears to be present in the peripheral, CMZ region of the retina is even weaker, and may in fact be a background signal (Fig. 3.7iB, F, J, N; 3.7iiB. F. J. N). αA-crystallin expression was observed in the central retina at all stages investigated, however, the pattern and level of this expression appeared to vary with developmental stage (Fig. 3.6iB, F, J, N; 3.6iiB. F. J. N). αA-crystallin expression in the central retina at HH18 was detected in all cells across the retina in a fibrillar fashion reminiscent of a membrane-bound protein, however, the strongest expression of the protein appeared to be localized to both the basal and apical surface of the retina, with expression largely decreasing in a gradient towards to middle of the neuroepithelium (Fig. 3.6iB). A similar expression pattern was observed in the retina at stage HH19 (Fig. 3.6iF), where αA-crystallin was expressed at comparable levels to that at HH18 (Fig. 3.6iB). By HH22, αA-crystallin was observed to be expressed at a higher level (Fig. 3.6iJ) than earlier in development, with apparently more cells at the vitreal surface exhibiting expression of the protein than before. A similar pattern and level of expression of αA-crystallin was maintained at both HH23 (Fig. 3.6iN) and HH24 (Fig. 3.6iiB), however, much more robust expression of the lens-associated protein was observed at the vitreal surface of the developing retina, in the presumptive ganglion cell layer (Fig. 3.6iiF). In addition to an increased expression level of αA-crystallin, more cells in this region appeared to be expressing the protein than at HH24.

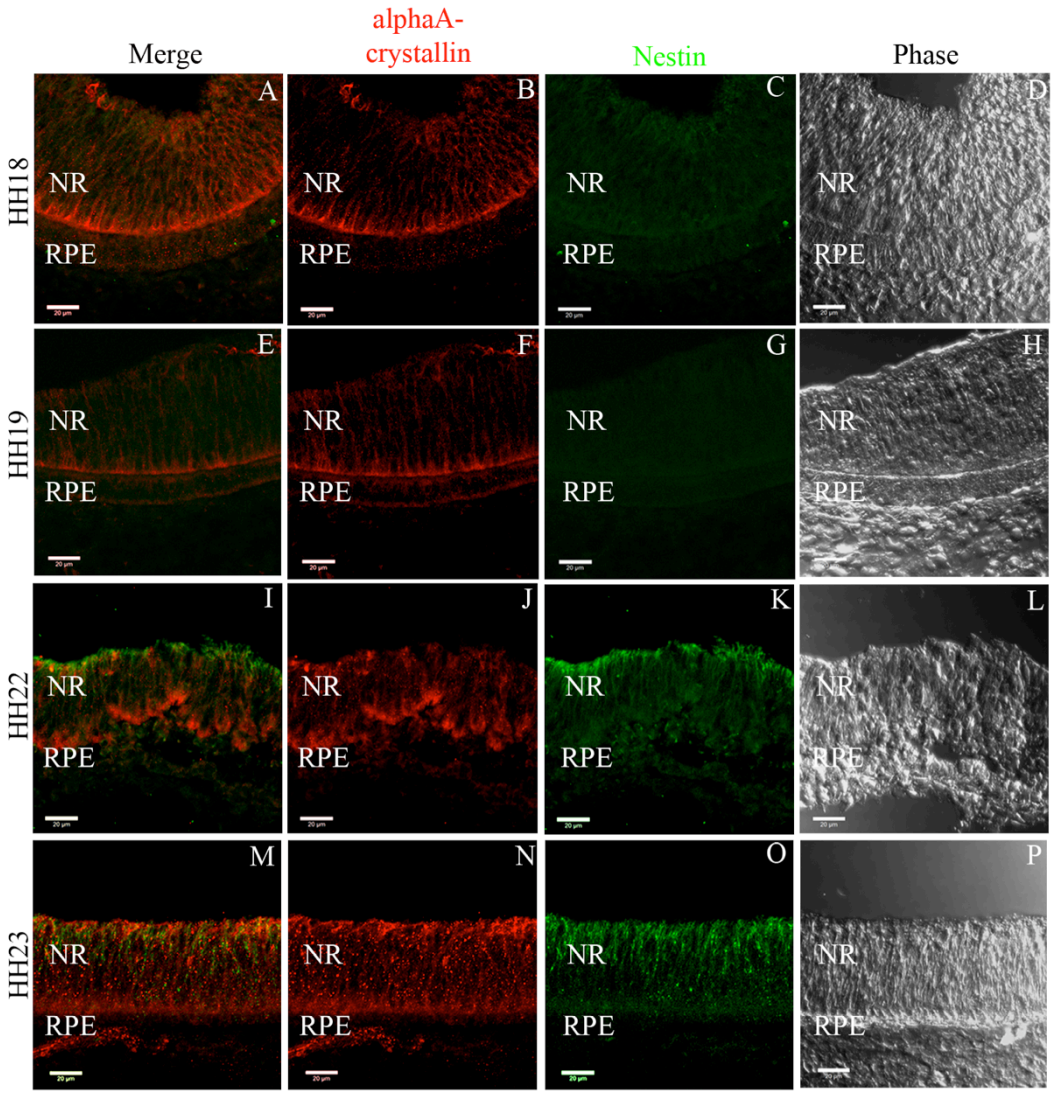


Fig. 3.6i
Developmental study of AACrystallin + Nestin expression in chick central retinal development
alpha-A-crystallin (red) was expressed in the neural retina (NR) at all developmental stages investigated HH18-HH23 (B, F, J, N) but was absent from the RPE at all stages. Expression appeared to be localised to the cytoplasms and membranes of the retinal cells with the highest expression of alpha-A-crystallin observed at the apical/basal surfaces at HH18-HH22 (B, F, J), but was observed across the neuroepithelium by HH23 (N), where the highest expression was localised to the presumptive ganglion cell layer at the vitreal surface. Nestin (green) expression was first observed at HH22 (K) in the cells at the vitreal surface of the retina, but was absent from RPE and neural retina at earlier stages: HH18 (C) and HH19 (G). Post-HH22, nestin expression explanded across the breadth of the neuroepithelium, with more cells in the middle of the neuroepithelium observed to express nestin by HH23 (O). Fluorescence digitally enhanced. Phase (D, H, L, P). Scale bars: 20uM.

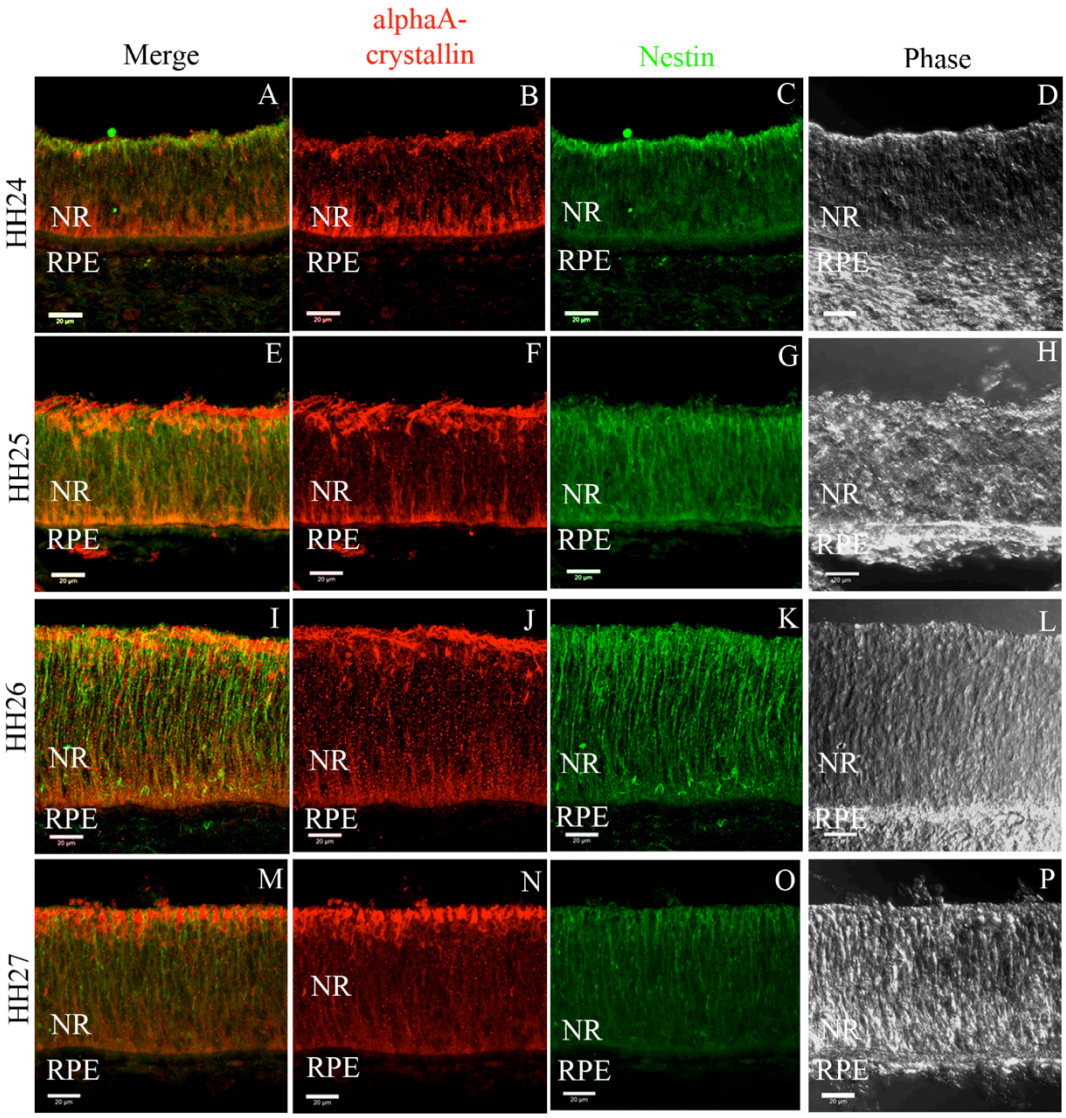


Fig. 3.6ii

Expression of alpha-A-crystallin and Nestin in the developing, central, chick retina. alpha-A-crystallin (red) was absent from the RPE cells at HH24-HH27 (B, F, J, N) but was robustly expressed in the neural retina (NR) at these stages. alpha-A-crystallin was expressed throughout the breadth of the neuroepithelium at all stages, with the highest level of expression gradually becoming localised to the presumptive ganglion cells layer of the retina, at the vitreal surface between HH24 and HH27 (B, F, J, N). Nestin (green) expression was most robustly expressed at the vitreal surface at stage HH24 (C) with some expression extending into the rest of the neuroepithelium. By HH25, all cells across the retinal neuroepithelium expressed nestin in a fibrillar pattern, which was perpendicular to the neuroepithelium (G). This pattern was maintained at stages HH26 (K) and HH27 (O). Merge (A, E, I, M), Phase (D, H, L, P) Fluoresence digitally enhanced. Scale bars: 20uM.

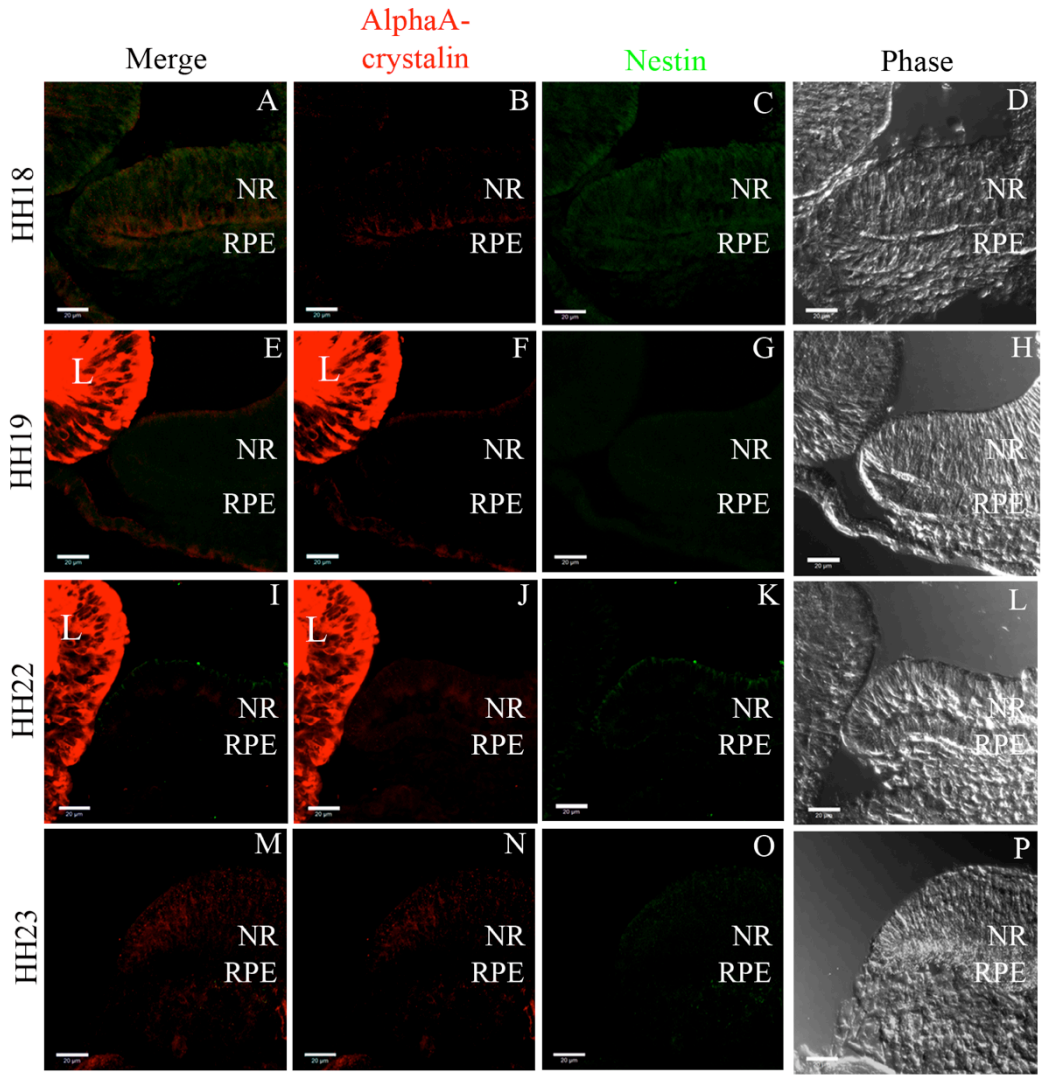


Fig. 3.7i

Developmental study of alpha-A-crystallin and nestin expression in chick, periperhal retinal development.

Alpha-a-crystallin (red) was robustly expressed in the lens F, J) at all stages but was also very weakly labeled in the CMZ at all stages (B, F, J, N), however, it is unclear whether or not this is background signal. No expression was observed in the RPE between HH18-HH23 (B, F, J, N). Retinal expression appeared to span the neural retina in a fibrillar fashion, similar to that observed in the central retina. Nestin (green) was not expressed in the CMZ RPE or neural retina (NR) at HH18-HH23 (C, G, K, O), which may suggest that this region is non-neuronal in identity. Merge (A, E, I, M), Phase contrast (D, H, L, P). Scale bars: 20uM.

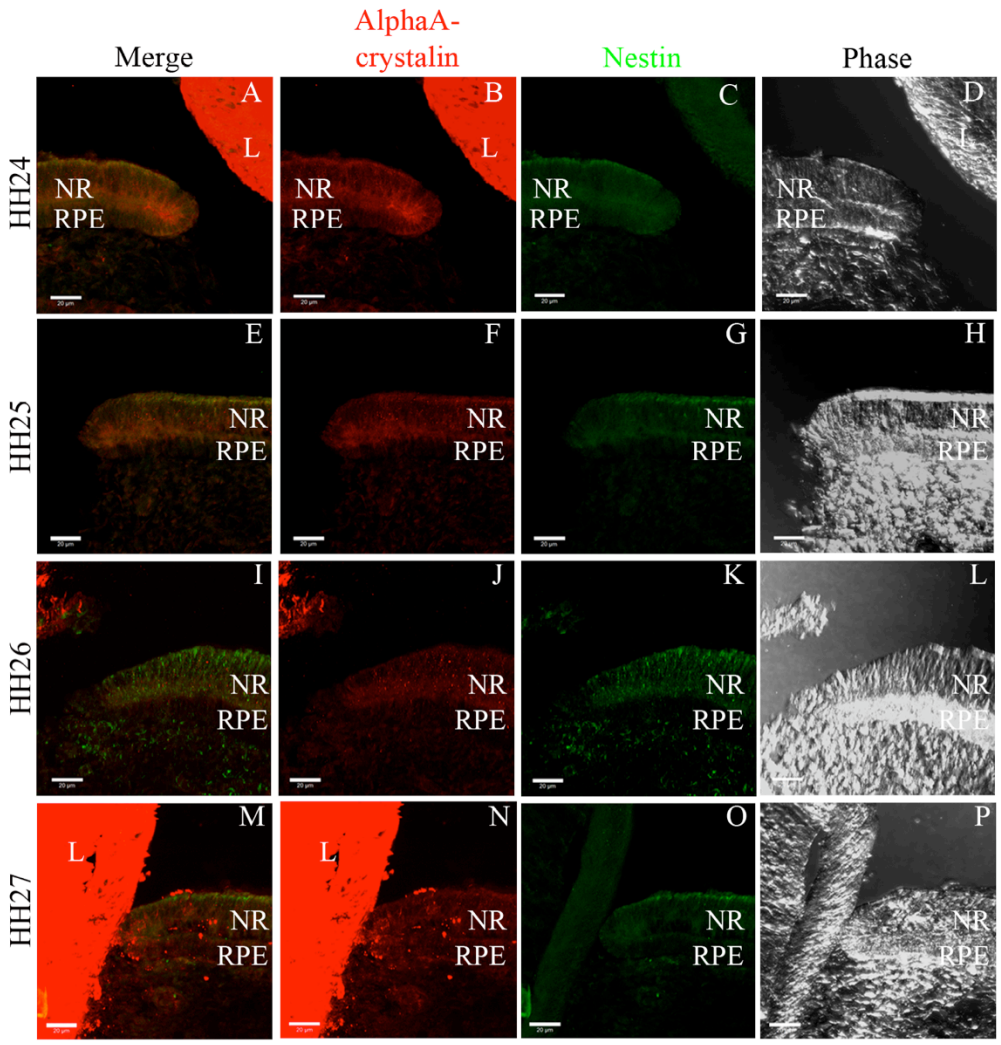


Fig. 3.7ii

Expression of alpha-A-crystallin and nestin in the periphery of the developing chick retina.

Expression of alpha-A-crystallin (red) was possibly observed in the neural retina at stages HH24-HH27, however, the signal was so weak it was unclear whether or not ir was background (B, F, J, N). In contrast the lens (L) was robustly expressed at all stages, as exhibited here at stages HH24 (B) and HH27 (N). No expression was observed in the RPE at any stage HH24-HH27 (B, F, J, N). Nestin expression appeared to be first detected at the vitreal surface of the neural retina at HH24 (C), and then gradually expanded across the neuroepithelium at stages HH25 (G) until HH26 (K) and HH27 (O), where nestin was observed in a fibrillar pattern across the entire neuroepithelium. No nestin labelling was observed in the RPE at any of these stages (C, G, K, O). Merge (A, E, I, M), Phase contrast (D, H, L, P). Scale bars: 20uM.

This pattern of expression was maintained at HH26 (Fig. 3.6iiJ), however, by HH27, almost all of the cells in the presumptive ganglion cell layer were expressing αA-crystallin at a very high level (Fig. 3.6iiM).

3.1.3.6 Nestin:

The expression of neural progenitor marker, nestin, was absent from the RPE at all stages investigated (Fig. 3.6iC, G, K, O; 3.6ii C, G, K, O; 3.7iC, G, K, O; 3.7ii C, G, K, O). Nestin was first detected in the central neural retina at HH22 (Fig. 3.6K), where it appeared to label the presumptive ganglion cell layer in a fibrillar fashion that extended from the vitreal surface towards the middle of the retinal neuroepithelium (Fig. 3.6iK). The protein was most robustly expressed at the vitreal surface and was reduced in a gradient towards the middle of the developing retina (Fig. 3.6iK). No nestin expression was detectable above the background signal at earlier stages of development, HH18 (Fig. 3.6iC), of HH19 (Fig. 3.6iG). A similar pattern of nestin expression observed at HH22 was also observed at HH23 (Fig. 3.6iO), however, the neuronal projections extending from the vitreal surface appeared to extend slightly further into the retina at this stage than at HH22 (Fig. 3.6iO, K respectively). By HH24 these projections appeared to span the entire breadth of the neuroepithelium (Fig. 3.6iiC), and at HH25 the level of expression of nestin was observed to increase (Fig. 3.6iiG). This robust expression of nestin across the developing retina was maintained at both HH26 (Fig. 3.6iiK) and HH27 (Fig. 3.6iiO), with the highest expression still exhibited at the vitreal surface. Expression of nestin at the peripheral CMZ region of the developing retina was even more difficult to analyse owing to the weak nature of the antibody labeling in this region, in addition to a high background fluoresence. It appeared as though nestin expression was largely absent from the neuroepithelium at earlier stages of development between HH18 and HH23 (Fig. 3.7iC, G, K, O), however, weak expression of the protein was detected at the vitreal surface in HH24 eyes (Fig. 3.7iiC). Expression of nestin appeared to extend across the neuroepithelium by HH25 (Fig. 3.7iiG) with the highest expression of the protein still

localized to the vitreal surface. This pattern of expression became more clear by HH26 where increased expression of nestin made this more clear (Fig. 3.7iiK). This pattern of nestin expression was maintained at HH27 (Fig. 3.7iiO).

3.1.3.6 Sox2:

Neural progenitor marker Sox2 was present in the retinal neuroepithelium at all stages investigated, but was not observed to be expressed in the RPE cells at any developmental stage between HH18, where the RPE remains unpigmented, and HH27 where the RPE has become heavily pigmented (Fig. 3.8i B, F, J, N; 3.8ii R, V, Z). Sox2 was robustly expressed throughout the neural retina at all stages, in the nuclei of most cells spanning the breadth of the neuroepithelium at stages HH18 to HH26 (Fig. 3.8i B, F, J, N; 3.8ii R, V, Z), where some cells in the retinal neuroepithelium expressed the transcription factor at lower levels, if at all. Many of these neural retinal cells were also observed to express Pax6 (Fig. 3.8i), particularly at the earliest stages of development where most cells of the neuroepithelium robustly expressed Pax6 (Fig. 3.8i A, C, E, G). However, a distinct layer of cells at the vitreal surface of the neural retina, were conspicuously not labeled for Sox2 expression (Fig. 3.8i B, F, J, N; 8ii R, V, Z). At HH18, many of these cells were also negative for Pax6 expression (Fig. 3.8i A-C), with perhaps only one Sox2 negative nucleus exhibiting a high level of expression of Pax6 at the vitreal surface (Fig. 3.8i A-C). The number of Sox2 negative cells in this layer which were subsequently observed in HH22 retina was greatly increased (Fig. 3.8i E-G yellow arrow), and this pattern was maintained at all later stages of development thereafter (Fig. 3.8i I-P; 3.8ii Q-Bi).

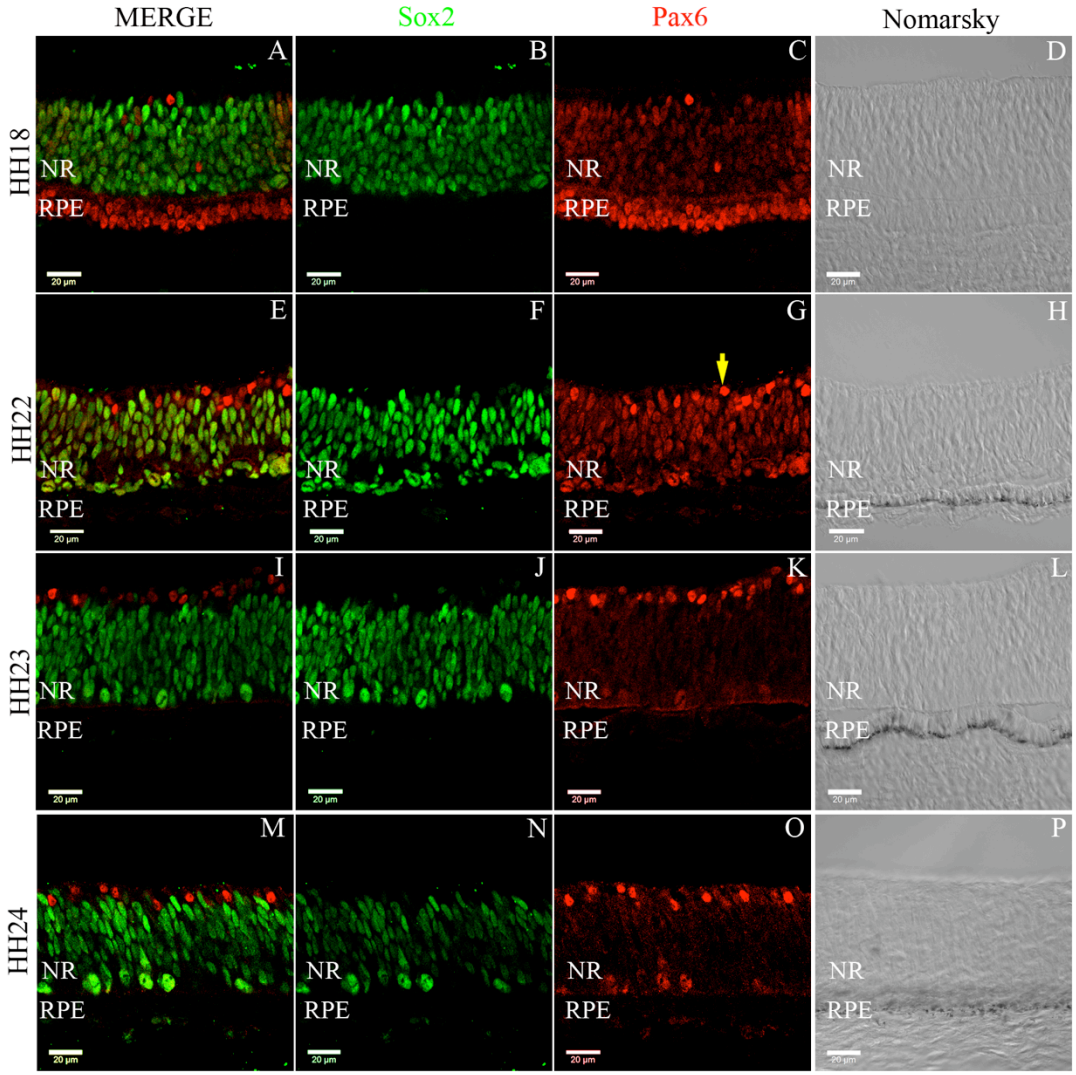


Fig. 3.8i

Developmental study of Pax6 + Sox2 expression in chick central retinal development (early stages).

Sox2 (green) was absent from the RPE at all stages, but was expressed in all retinal cells between the stages of HH18 and HH24 (B, F, J, N) except for a single layer of cells at the vitreal surface, which often expressed Pax6, particularly after HH18 (C, G yellow arrow, K, O). Pax6 was only observed in the RPE at HH18 (C), however, it appeared to be largely absent from the RPE at all stages thereafter (G, K, O), which is surprising given that Pax6 expression appears to be important for the maintenance of the potential for transdifferentiation. Pax6 was robustly expressed in all neural retinal cells at HH18 and HH19 (C, G) but appeared to be down-regulated in most retinal cells at HH23 (K) and HH24 (O) where the level of expression was lower. Many Pax6 positive cells also expressed Sox2 in the retina, indicating the presence of retinal progenitors, particularly at early stages (A, E). Sox2 negative, Pax6 positive cells at the vitreal surface indicate cells which have begun neurogenesis, mostly likely towards a ganglion cell fate given their localisation (A, E, I, M). Fluoresence digitally enhanced. Scale bars: 20uM.

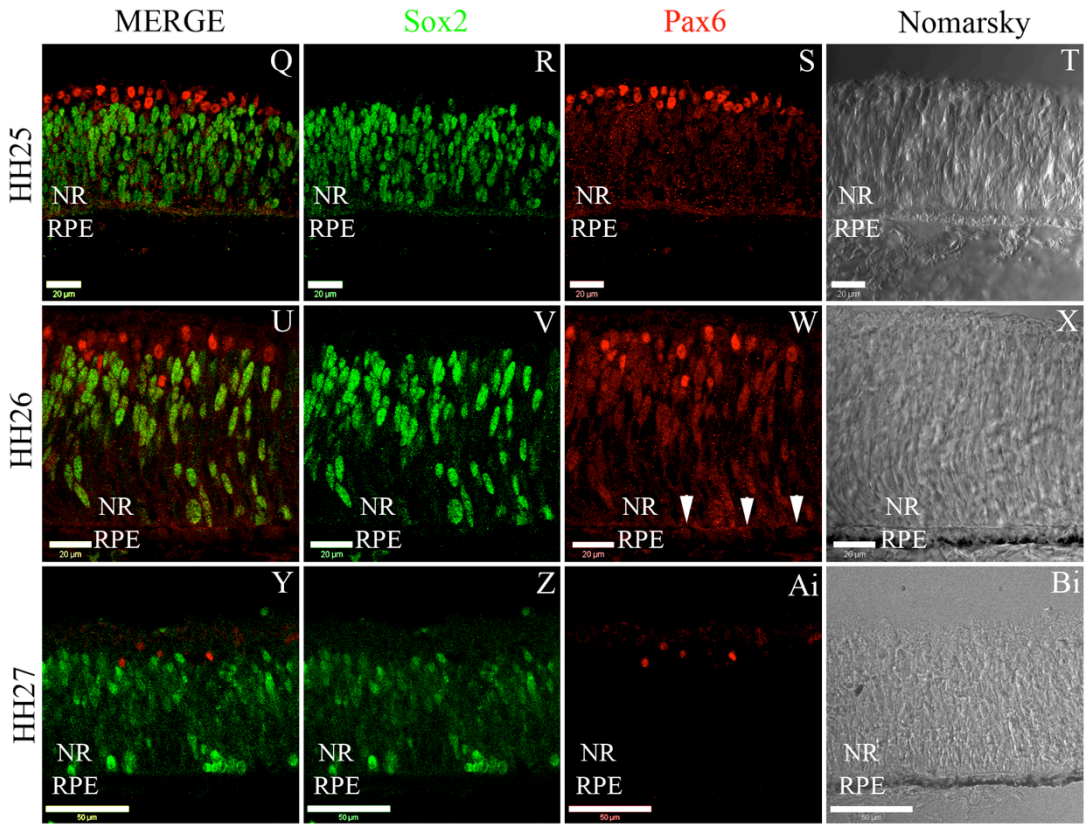


Fig. 3.8ii

Developmental study of Pax6 + Sox2 expression in chick central retinal development (later stages).

Pax6 (red) expression was observed in the neural retina (NR) at all stages HH25 to HH27 (S, W, Ai). Highest expression was observed in those retinal cells closest to the vitreous, however, some weaker expression was also maintained throughout other cells of the retina at HH25 (S) and HH26 (W). Many cells within the rest of the retina did not express Pax6 by HH27 (Ai), where Pax6 expression was confined to the nuclei of cells in the presumptive ganglion cell layer. Cells expressing the highest level of Pax6 in this vitreal surface region did not co-express Sox2, indicating a differentiated state (Q, U, Y). Other cells expressing Pax6 in the retina did express Sox2, implying a retinal progenitor cell identity. Sox2 was expressed throughout the retina, except at the vitreal surface, at all stages (R, V, Z), however, by HH26, some cells appeared to have down-regulated Sox2, with only weak labeling observed in some cells (V, Z). No Sox2 expression was observed in the RPE at any stage (R, V, Z), which was generally also the case for Pax6 (S, Ai). However, a few RPE cells at HH26 (W, white arrowheads), but not all RPE cells did appear to express limited Pax6. Fluoresence digitally enhanced. Scale bars: 50uM.

3.1.3.7 Pax6:

Interestingly, despite all cells of the neural retina expressing Pax6 at early stages of development HH18 and HH22 (Fig. 3.8i C, G), many nuclei throughout the neuroepithelium were observed to down-regulate Pax6 expression by HH23 (Fig. 3.8i K), with much weaker expression observed in this region at subsequent developmental stages (Fig. 3.8i K, O; 3.8ii S, W). Only the cells at the vitreal surface maintained robust expression of the transcription factor at later stages of development (Fig. 3.8i K, O; 3.8ii S, W). Interestingly, no Pax6 expression was detected in the neural retina at HH27, and only weak expression of Pax6 was observed in the vitreal surface layer at this stage (Fig. 3.8ii Ai).

Surprisingly, expression of Pax6 was only observed throughout the RPE at the earliest stage of development, before the RPE had pigmented, at HH18 (Fig. 3.8i C, D). After this stage, Pax6 expression appeared to be absent from the majority of the central RPE following pigmentation (Fig. 3.8i G, K, O; 3.8ii S, W, Ai). However, some weak expression of Pax6 was detected in disparate nuclei in a small number of cells of the RPE, suggesting that the expression of Pax6 may be variable across the monolayer (Fig. 3.8ii W white arrowheads).

Flatmount, *en face* labeling for Pax6 expression in RPE monolayers at various developmental stages did exhibit variability in the expression of Pax6 across the RPE sheet (Fig. 3.9). All pigmented nuclei did appear to express Pax6 at HH24 (Fig. 3.9A, B), however, by HH25, the expression of Pax6 across the RPE monolayer was observed to be very variable (Fig. 3.9 C-R). Some regions of RPE appeared to express Pax6 in most RPE nuclei (Fig. 3.9C), however, other regions did not exhibit any Pax6 expression (Fig. 3.9E). This difference in the expression of Pax6 did not appear to correlate with the level of pigmentation in RPE cells given the fact that both regions displayed similar levels of pigment (Fig. 3.9D, F). A similar pattern was also observed at HH27 (Fig. 3.9G-J) where some regions of RPE displayed robust Pax6 expression in the majority of cells (Fig. 3.9G), with other regions displaying only very weak labeling for the transcription factor (Fig. 3.9I).

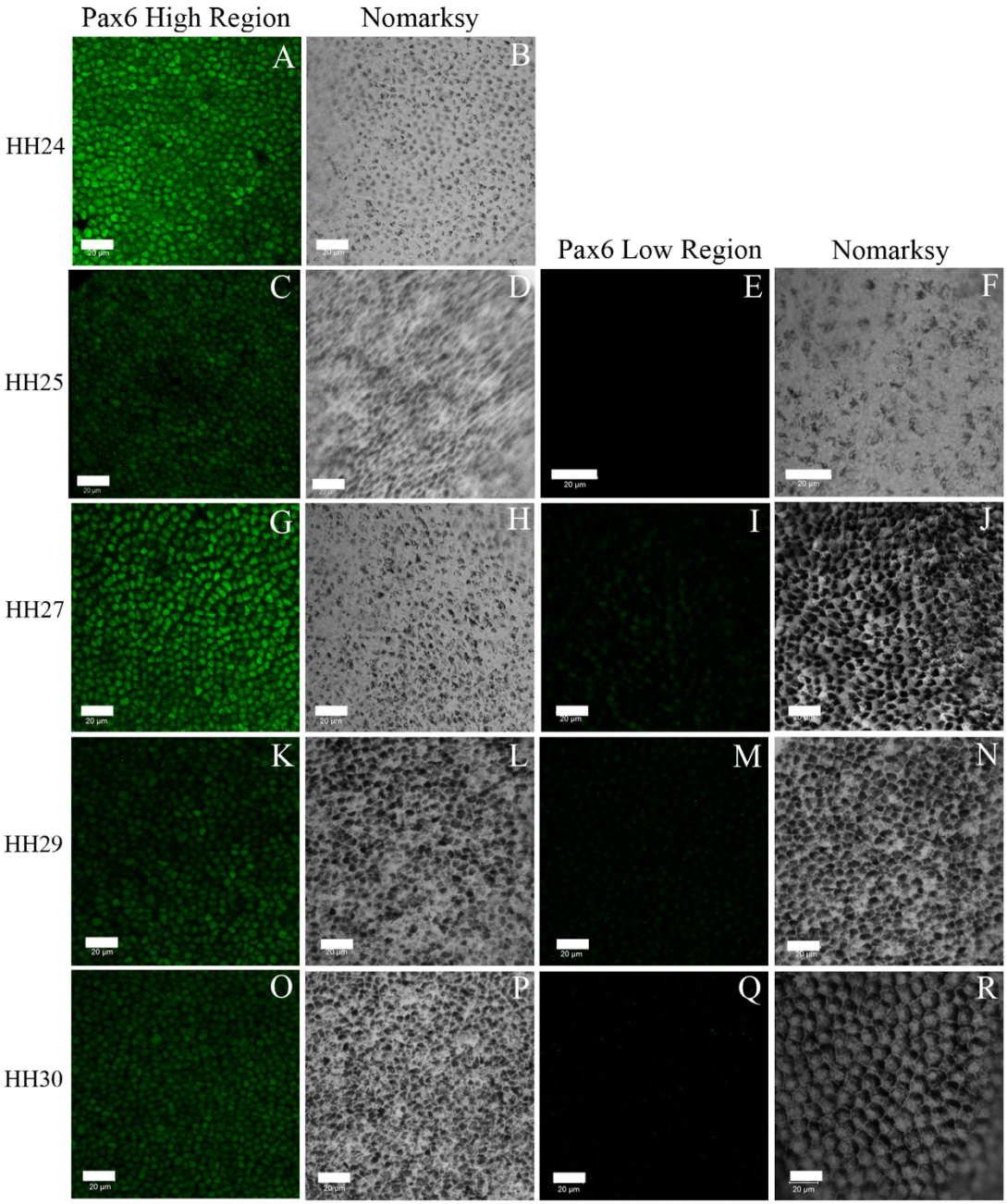


Fig. 3.9

Developmental study of Pax6 expression *en face* in chick RPE monlayer development. Pax6 exhibited a variable expression pattern across different areas of dissected fragments of chick RPE at different developmental stages. Pax6 was expressed in some regions of RPE at all stages investigated, from HH24 to HH30 (A, C, G, K, O). However, beyond HH24, other regions of the same RPE sheets had barely detectable levels, if any expression, of Pax6 (E, I, M, Q). This difference in Pax6 expression did not appear to be reflected by the corresponding level of pigmentation, given that both high expression regions (B, D, H, L, P) and low expression regions (F, J, N, R) exhibited very variable levels of pigmentation. Scale bars: 20uM.

In this instance, the region with less pigmentation displayed less intense pigmentation (Fig. 3.9H) than the region with lower Pax6 expression (Fig. 3.9J). The variability in the expression level of Pax6 was maintained at both HH29 (Fig. 3.9K-N) and HH30 (Fig. 3.9O-R) where some regions displayed relatively uniform populations of Pax6 positive cells (Fig. 3.9K, O), whereas others exhibited weak (Fig. 3.9M), if any (Fig. 3.9Q) expression of Pax6. This variability in Pax6 expression did not appear to correlate with the level of pigmentation within the RPE at later stages, given the comparable levels of pigmentation between regions with differing expression levels of Pax6 (Fig. 3.9L, N, P, R).

3.1.4 Discussion:

3.1.4.1 HuD & Islet1:

Expression of differentiated retinal cell markers was observed in the central retina of chicken eyes from very early stages of development. Both HuD and Islet-1, which are known to label: ganglion/amacrine (Sakami et al., 2008), and ganglion cells (Elshatory et al., 2007, Sakami et al., 2008), respectively, were expressed at a similar stage of development. This indicates that ganglion cells begin the develop around HH18/19 at the vitreal surface, which is to be expected given that this cell type is reportedly the first differentiated cell type to emerge in the developing retina (Prada et al., 1992, Prada et al., 1991, Cepko et al., 1996, Kahn, 1973). In addition, positive cells were localized to the area of the retina where the ganglion cell layer is expected to develop, as in adult retina. This early stage of expression of HuD is consistent with its role in the neuronal-specific RNA-processing in the differentiation of both amacrine and ganglion cells, and the fact that the most robust expression was observed from HH23 is consistent with the birth of most retinal ganglion cells in the retina between E3 and E8 (Kahn, 1973). Some HuD expression was also observed in the middle of the retinal neuroepithelium around HH24 onwards. These are most likely to be: differentiating amacrine cells that are not labeled by Islet-1, or migratory ganglion cells that have been born in this

region before re-locating to the vitreal surface. It has been reported that post-mitotic, pre-migratory, retinal ganglion cells, which are positive for RGC marker, RA4, exist in this region, and that Otx2 has an important role in the migration of the cells, given that it appears to be expressed in migrating cells during their migration only (Bovolenta et al., 1997). By HH27, only very few cells in the middle of the neuroepithelium are observed to express HuD, which suggests that the majority of cells have completed their migration.

In addition to labeling retinal ganglion cells, Islet-1 has also been implicated in bipolar cell development (Elshatory et al., 2007), however, it is unlikely that the positive cells observed are bipolar cells, given that they are located at the vitreal surface, in addition to the fact that bipolar cells are known to differentiate at later stages of development (Prada et al., 1991).

It is unsurprising that Islet-1 is observed at a slightly earlier developmental stage than HuD, given that the two stages are very close together in development, and some developmental overlap is likely in such a dynamic system. The numbers of cells expressing both of these proteins was observed to gradually increase with progressive development, indicating that more ganglion cells differentiate with on-going development. Expression of both these markers is highest in the central-most retina and spreads in a central to peripheral gradient with development, indicating that the central retina is developmentally more mature than that in more peripheral regions. HuD and Islet-1 were not observed in the CMZ region of the retina at the stages analysed, which may suggest that this region is yet to begin neurogenesis, or may indicate that this region is phenotypically different in its cell make-up than central retina. This seems likely given that this region is reported to contain a population of retinal stem cells, which are able to proliferate and differentiate to both continually add cells at the periphery of the retina throughout the life of animals such as chickens, or teleosts, in addition to regenerating the retina under certain circumstances (Spence et al., 2007a, Kubo and Nakagawa, 2008, Kubota et al., 2002, Susaki et al., 2008, Wehman et al., 2005, Wilson et al., 2007, Reh and Fischer, 2001, Tropepe et al., 2000, Spence et al., 2004). However, the size

and potential of this region appears to vary between different species, and no reports of such a zone have been made in humans to date.

The lack of expression of HuD or Islet-1 in the RPE suggests that the markers are neuron specific and that the RPE does not exist in a state where they are already pre-disposed towards a neuronal fate. The early onset of both HuD and Islet-1 expression in the neuroepithelium would suggest that they would be potentially good markers for identifying transdifferentiation, as they should only be observed transdifferentiated RPE retina, and not in the RPE.

3.1.4.2 FGF-R1:

The suggestion that a down-regulation of FGFR-1 is responsible for a loss in transdifferentiation (Spence et al., 2004) in response to bFGF appears not to be true as the receptor expression was maintained at an apparently steady level in both retinal cells and RPE cells throughout development. This is consistent with previous reports which have shown that transcriptional levels of FGFR-1 remain relatively constant over the period when bFGF is reported to initiate transdifferentiation in the RPE, with one study even suggesting that FGFR-1 expression levels in the RPE slightly increasing after E4, the opposite of what would be expected (Tcheng et al., 1994, Ohuchi et al., 1994).

However, in order for bFGF to bind its high-affinity receptor, it requires the presence of co-factors in the form of heparin-sulfate proteoglycans. Free heparin and cell surface bound heparin-like molecules have been shown to act as a low-affinity binding sites for free bFGF that are critical for the further binding to the high affinity receptor, FGFR-1 (Yayon et al., 1991, Ornitz et al., 1992, Kim et al., 2003, Forsten-Williams et al., 2008, Cirillo et al., 1990). Cells lacking in heparin as a result of heparitinase-treament fail to bind bFGF, which illustrates that heparin is necessary for proper binding of bFGF. It also aids the dimerization of bFGF oligomers which may be important for its function (Ornitz et al., 1992). In addition, bFGF has been shown to be very unstable in culture and heparin has shown to potentiate its

effects in culture either through stabilization of the free protein, thereby preventing its degradation, or through aiding the binding of bFGF to its high-affinity receptor (Chai and Morris, 1999, Caldwell and Svendsen, 1998, Caldwell et al., 2004, Furue et al., 2008). It is possible that changes in the levels and localization of heparin during the development of the RPE may change its sensitivity to bFGF signaling. It has been previously suggested that heparin must have roles in the embryonic chicken retina, other than its role as an anti-clotting agent (with which it is often associated), owing to the fact that there is no vasculature in the embryonic retina (Mascarelli et al., 1987). Regulation of growth factor signaling could be the reason for the presence of heparin-like molecules in the developing retina. Indeed, it has already been shown that heparin-proteoglycans in the inner-limiting membrane (ILM) are able to regulate neuronal development through binding of bFGF, which in turn promotes axonal outgrowth and in developing neurons (Chai and Morris, 1999). Similarly, heparins have been shown to direct the function of FGFs in the axonal targeting of new neurons (Walz et al., 1997).

Changes in the extracellular matrix, in particular, laminin, might also potentiate the effects of bFGF and heparin (Ren et al., 2006, Yu et al., 2007, Otaegi et al., 2007, Araki et al., 2002). Laminin has been shown to be an inducer of retinal regeneration via transdifferentiation of the RPE in newts, possibly as a result of the potentiation of the effects of bFGF known to induce regeneration (Reh et al., 1987, Araki, 2007, Mitsuda et al., 2005, Susaki and Chiba, 2007). Newt RPE cells grown on laminin are also regularly observed to undergo neuronal differentiation. An important part initiator in the initiation of retinal regeneration is thought to be the migration and attachment of RPE cells to the vascular membrane, which in frogs is enriched with laminin. Therefore, through potentiation of the effects of bFGF, this could initiate the onset of transdifferentiation (Reh et al., 1987). It is possible that during development, changes in the levels of these various co-factors within the RPE are able to regulate its response to bFGF, and thereby limit its ability to undergo transdifferentiation.

3.1.4.3 Chx10:

Chx10 is often used as a marker of multi-potent state in both undifferentiated cells of the optic cup, as well as the retinal progenitor cells of the immature retina (Burmeister et al., 1996, Rowan et al., 2004, Horsford et al., 2005, Chen and Cepko, 2000). Indeed, Chx10 is thought to be critical in conferring a neuroretinal identity, without which retinal cells are observed to transdifferentiate towards a pigmented, RPE-like phenotype (Rowan et al., 2004). Therefore, it was thought that the embryonic RPE might express the Chx10 transcription factor at early stages, and perhaps subsequently lose expression at a stage that would coincide with a loss in the potential for transdifferentiation in response to bFGF. In doing so conferring a retinal potential upon the RPE, which is subsequently lost. Despite some signal being identified within the RPE at all stages of development investigated, the pattern of expression was uncharacteristically non-nuclear in both the RPE and adjacent retina. The pattern of expression did not resemble that expected for a transcription factor, and therefore, it is likely that this antibody had labeled another antigen in a non-specific manner. This would mean that this antibody is unsuitable for use in chicken embryos.

3.1.4.4 αA-crystallin:

αA-crystallin is normally associated with the formation of the lens, given that it makes up the majority of the structural protein that comprises the lens via a complex with αB-crystallin in a 3:1 ratio (Cvekl et al., 1995, Das et al., 1999, Fujii et al., 2003, Sun and Liang, 1998). It was previously thought that αA-crystallin was restricted to the lens whereas αB-crystallin was expressed in other tissues (van Boekel et al., 1999). However, in addition to robust staining in the lens, weaker labeling was also observed in the developing retina. This was initially assumed to be non-specific binding, however, it has been reported that αA-crystallin has another function besides its role as a structural protein in the lens. αA-crystallin has been shown to act as a molecular chaperone, which is able to protect against the

aggregation of proteins that have been damaged by heat, stress or U.V. (Andley et al., 1996, Cherian-Shaw et al., 1999, Das et al., 1999, Kundu et al., 2007, van Boekel et al., 1999).

It is possible therefore that αA-crystallin is expressed in developing retinal cells in order to provide the cell with this chaperone function. It could be that the high metabolic stress associated with the rapid proliferation of retinal progenitors, in addition to the differentiation of various retinal cell types, requires the chaperone function of αA-crystallin in order to protect the cells from harm. This is perhaps an even more attractive hypothesis given that the highest expression appears to be at later stages, after HH25, at the vitreal surface which is where the newly differentiated retinal ganglion cells, the first cell type to differentiate (Prada et al., 1991), are located. At the far periphery of the retina, only very weak labeling for αA-crystallin was observed, however, this did not increase at the vitreal surface with development like that of the central retina, which again suggests that the expression of αA-crystallin is somewhat associated with the differentiation of specific cell types in the retina, given that the central to peripheral gradient in neurogenesis within the retina would account for the lack of differentiation in this region (Prada et al., 1991). In addition, the fact that the chicken eye has a region of retinal stem cells at the CMZ would mean that little differentiation is likely taking place in this region. Despite the apparent labeling of αA-crystallin in the retina, no labeling was observed in the RPE at any stage as initially expected.

3.1.4.5 Nestin:

The early neuronal marker nestin was not expressed in the RPE at any stage and it therefore unlikely to indicate a loss in capacity for transdifferentiation as a result of the maintenance of a neural progenitor-like state. The fact that it was expressed in the neuroepithelium beyond HH22 could however mean that nestin could potentially be a good marker of neuroepithleial specification following transdifferentiation of the RPE. The expression appeared initially to be located at the vitreal surface of the retina, and gradually

expanded across the breadth of the retina as development progressed. It is unclear why this pattern of expression exists, however, initially, it is possible that only differentiating neuronal cells at the vitreal surface expressed the protein and were extending processes into the rest of the retina. At later stages, other differentiating cell types may also begin to express nestin as they develop, which gives the appearance of labeling across the breadth of the retina. The majority of expression at the far periphery of the retina was confined to the vitreal surface at later stages, with no labeling obvious at the earliest stages, which may reflect a delay owing to the central to peripheral gradient in neurogenesis, given the positive labeling in the central retina at these stages (Prada et al., 1991).

It is perhaps surprising that nestin expression appears to become more robust in the retina at later stages of development given that it is usually associated with the presence of retinal progenitor cells, of which there should be more at earlier stages before cells begin to differentiate into specific retinal cell types. The reason for this spatio-temporal expression pattern is unclear, however, it may reflect a specific progenitor state, possibly post-mitotic cells before they fully differentiate, given the apparently high expression at the vitreal surface at later stages where ganglion cells are developing. However, further investigation would be necessary to confirm this.

3.1.4.6 Pax6 & Sox2:

As previously reported, the expression of Sox2 was not observed in the RPE at any stage. This would suggest that the maintenance of a multi-potent state in the RPE is not as a result of a maintenance of a retinal progenitor/stem cell-like state, as one would expect Sox2 to be expressed in accordance with its role in maintenance of this state (Ma et al., 2009). In addition, it has been reported that a down-regulation in the family of Sox genes, of which Sox2 is a member, in the outer layer of the optic cup, is necessary for the proper development of the RPE (Ishii et al., 2009). Ectopic expression of Sox2 in the presumptive RPE leads to a loss in the RPE phenotype, which is replaced by cells of a neuronal phenotype, i.e.

transdifferentiation (Ishii et al., 2009, Ma et al., 2009). Sox2 expression in the retina is usually only expressed within amacrine cells and specific Müller glial cells in the adult retina (Lin et al., 2009), but was observed at all stages in the embryonic retina. This is consistent with its critical role in the maintenance of retinal progenitors, as well as neural competence of these cells.

For the most part, Sox2 expression in the majority of the retina was co-expressed with Pax6. The combined expression of Sox2 and Pax6 has been reported to be critical for the neural competence of the developing retina, with knock out of Sox2 leading to an increase in Pax6 expression, and a subsequent loss of neural progenitors in the retina, which eventually re-differentiate as non-neurogenic, ciliary epithelium (Matsushima et al., 2011). This accounts for the large number of cells across the developing retina that are observed to co-express the two transcription factors. The regulation of differentiation of retinal cell types appears to rely on the shared interactions between Sox2 and Pax6 on a number of occasions. It has been reported that ectopic Sox2 expression is able to increase the expression of Pax6 via its promoter in *in vitro* expression studies in retinal cell lines (Lin et al., 2009). Additionally, ectopic Sox2 expression has been shown to increase the population of amacrine cells in the developing inner nuclear layer, within the embryonic retina (Lin et al., 2009). Conditional down-regulation of Pax6 in developing retinal progenitor cells lead to the exclusive production of amacrine cells in the retina (Oron-Karni et al., 2008), which would suggest a critical role for Pax6 in regulating the fate of differentiating retinal progenitors, possibly via its interaction with Sox2. At later stages of development, following pigmentation of the RPE in the embryonic chick eyes, not all Pax6 expression in the retina was restricted to Sox2 positive retinal progenitors, but was most robustly expressed in cells at the vitreal surface, which were Sox2 negative. This would suggest that these cells are differentiating into specific retinal cell types, which do not require maintenance of Sox2 expression.

Given that Sox2 maintenance is required for amacrine cell development, is it likely that these differentiating cells are newly born ganglion cells, which are reported to express Pax6 at equivalent stages of development in humans (Nishina et al., 1999). This hypothesis is

supported by the fact that the appearance of these Pax6 positive cells at the vitreal surface corresponds to the period where the majority of ganglion cells are starting to be born (Prada et al., 1992, Prada et al., 1991, Kahn, 1973), as well as the fact that these are the stages where HuD starts to be expressed at the vitreal surface. In addition, Islet-1 positive cells are also located in this region. Pax6 has been reported to have a role in the onset of the expression of a number of other retinal cell types, which accounts for its continued expression across the developing retinal neuroepithelium. At later stages, it is likely that this expression becomes more restricted to the ganglion cell layer because more cells across the neuroepithelium are undergoing differentiation. For example, it has been suggested that Pax6 down-regulation is necessary for photoreceptor differentiation (Oron-Karni et al., 2008), which would tally with the apparent lack of Pax6 expression in cells located in the ONL, immediately adjacent to the RPE, at later stages of development.

Contrary to previous reports, Pax6 expression in the RPE was observed to be maintained at stages far beyond HH25, which had previously been reported to be the point at which Pax6 was down-regulated (Spence et al., 2007b). It was unclear as to why this wasn't apparent in sectioned eye tissue, but was very clear in some regions of RPE monolayers viewed *en face*. It will be necessary to develop better dissection techniques in order to investigate whether this down-regulation of Pax6 was specific to a particular region of the Pax6, or merely a reflection of the non-uniform development of the RPE sheet. If indeed the down-regulation of Pax6 in the RPE is restricted to a particular region, this could yield clues as the signaling molecules involved in the on-going maturation of the RPE. It is likely that these would also be involved in the restriction of the capacity for transdifferentiation, given the importance of Pax6 expression in transdifferentiation (Spence et al., 2007b, Azuma et al., 2005a, Kuriyama et al., 2009b). Pax6 expression was previously reported to coincide with the loss in capacity for transdifferentiation (Spence et al., 2007b), which would support this idea. It is possible that Pax6 expression could be up-regulated in the short period between dissection of the RPE monolayers and fixing. A similar effect is observed in the newt upon removal of the RPE sheet from the adjacent choroidal tissue (Kuriyama et al., 2009a). An up-

regulation of Pax6 in the RPE of regenerating newt retina is thought to be necessary for bFGF-mediated transdifferentiation, and without its expression, bFGF is ineffective (Kuriyama et al., 2009a). This investigation has demonstrated that chicken RPE cells retain the expression of Pax6 beyond that previously reported. Could it therefore be that the capacity for transdifferentiation is also retained beyond the point which was previously reported? This is of particular importance given the central role that Pax6 appears to play in transdifferentiation.

This variation in the expression of the transcription factor Pax6 was unexpected, but perhaps not surprising given that the developmental time-frame over which this investigation took place is a matter of days. In the rapidly changing environment of the developing eye, it is therefore likely that there will be a degree of developmental overlap in the spatio-temporal expression of various factors. The concept of biological variation in a structure as complex as the developing eye is perhaps to be expected more readily than other structures, given the complicated array of growth factor signaling gradients, and the sensitivity of each particular cell to each of these signals, involved in the proper formation of the eye. It is likely that cells of any particular ocular tissue will not develop in a completely homogenous manner given that each cells position within the eye will experience different micro-environmental signaling triggers, which subsequently dictate a unique developmental program, possibly even different from the most adjacent retinal cells, even those of the same apparent lineage, such as the RPE. This is especially likely for multi-functional transcription factors like Pax6, whose complex expression profile controls numerous developmental processes. Variation in the RPE is particular interesting given that developmental signaling can in theory occur throughout the RPE monolayer, via the various GAP junctions that are known to link these cells together. It is entirely possible that this could lead to localized build up of particular signaling factors that subsequently diffuse throughout the RPE monolayer, with RPE cells responding in a heterologous pattern dependent on their interaction with them.

3.2 Chick RPE transdifferentiation in a humanized culture system:

3.2.1 Introduction:

In order to use the chicken model of RPE transdifferentiation as a model with which to study the potential for human RPE transdifferentiation, it must first be confirmed that the phenomenon can be replicated sufficiently well so that it can be investigated. In addition, it is important to ascertain whether or not transdifferentiation can still be induced in a human cell optimized system, using the human growth medium commonly used to grow human RPE cells *in vitro*, as well as the human, recombinant bFGF which is proposed to be the trigger for the onset of transdifferentiation.

The standard growth medium used to grow the human fetal RPE cells is known as HESC medium (human embryonic stem cell medium). This medium contains a high concentration (20%) of an undefined, commercially available, serum replacement (Vugler et al., 2008). It is necessary to confirm that the contents of this serum replacement do not have an inhibitory effect on the onset of transdifferentiation, as this could potentially be a reason why no obvious evidence for classical transdifferentiation was observed in the human fetal RPE in response to bFGF. Similarly, it was necessary to confirm that the embryonic chick RPE could both: survive in a medium that has been osmotically optimized for human cells, and transdifferentiate in response to a human recombinant bFGF at a concentration of 100ng/ml. Additionally, it was necessary to characterize the changes in gene expression that occur as a result of the phenotypic change. This will confirm whether or not any observed transdifferentiation exhibits the classical standards which have been reported. This includes the identification of the various cell types resulting from transdifferentiation of the RPE, which will provide a better understanding of how closely the formation of a new neuroepithleium resembles the development of the native presumptive retina. Lastly, if the process of RPE transdifferentiation is to be employed in the production of different cell types

for transplantation and repair of retinal degeneration, it is necessary to confirm whether or not these types of cells are present in the novel retina.

3.2.2 Materials & Methods:

3.2.2.1 RPE isolation & culture:

Embryonic chick RPE explants were dissected from chick embryos at HH24 (approximately E4) and cultured in a standard, non-adherent, transdifferentiation culture system, +/-bFGF (100ng/ml), for 7 days, as described in chapter 2.

3.2.2.2 Immunohistochemistry:

Immunohistochemical analysis was performed as described in chapter 2.

3.2.3 Results:

Transdifferentiated RPE explants quickly formed pigmented, sphere-like aggregates within 24 hours of culture, which subsequently began to lose their characteristic pigmented

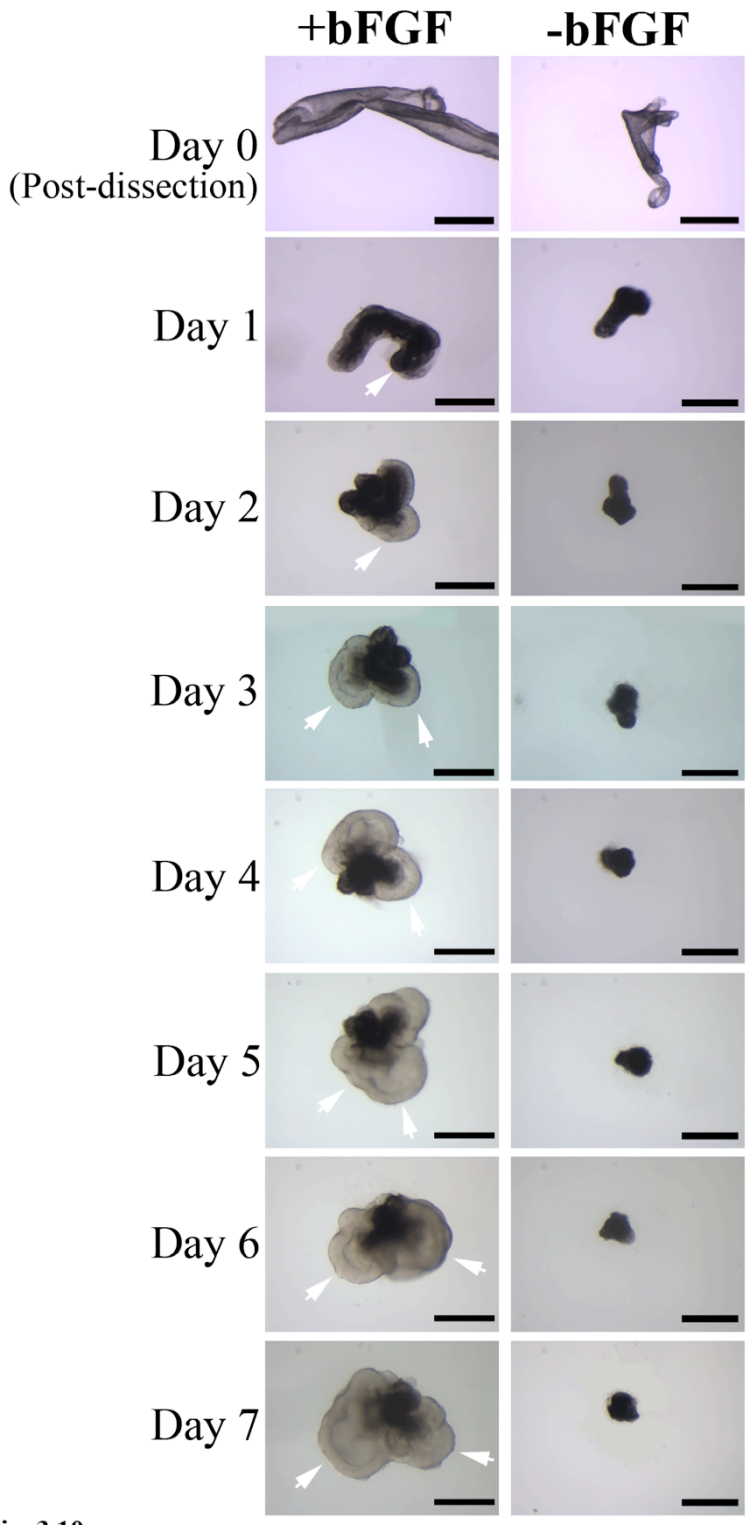


Fig. 3.10
The progression of transdifferentiation of Chick RPE HH24 treated with bFGF - transdifferentiation with a retention of some pigmentation - Embryo 1.
bFGF treated RPE explants in HESC medium exhibited the development of neuroepithelium loops which were absent from untreated explants, over a period of 7 days. Some pigment was retained in most bFGF treated explants and this pigmented region retained a similar size throughout the culture period. This would suggest that the neuroepithelial loops grow as a result of the expansion of neural progentiors produced via transdifferentiation, and not as a result of further transdifferentiation of more RPE. White arrows indicate neuroepithelial loops. Scale bars: 200uM

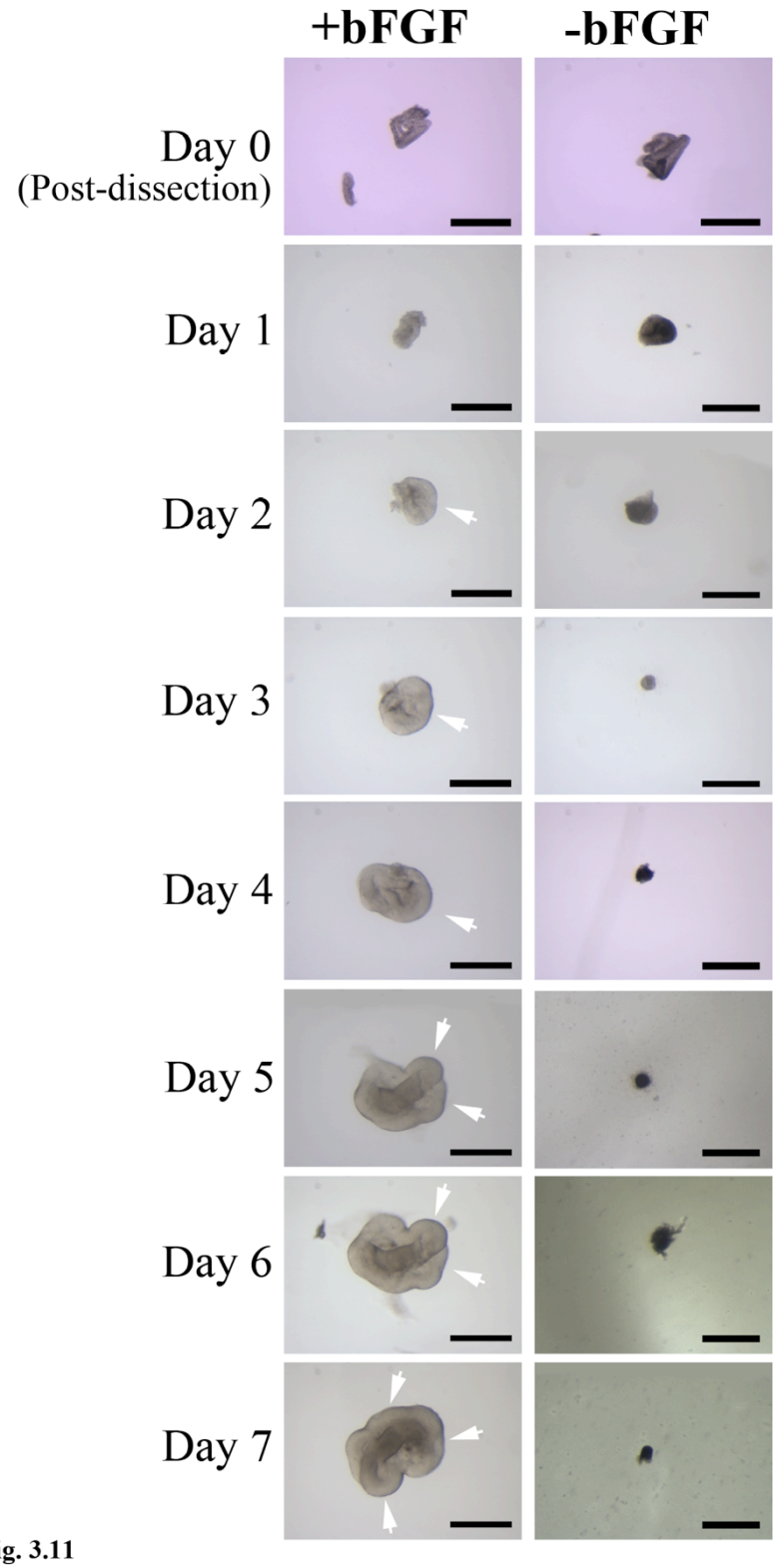


Fig. 3.11
The progression of transdifferentiation of Chick RPE HH24 treated with bFGF - transdifferentiation with no retention of pigmentation - Embryo 2.
bFGF treated RPE explants in HESC medium exhibited the development of non-pigmented, neuroepithelium loops which were absent from untreated explants. In this rarer instance, no pigmentation was visible following transdifferentiation. This confirms that transdifferentiating RPE cells are the source of the neuroepithelial loops, rather than contaminating reitnal progenitors which could proliferate in response to bFGF treatment. White arrows indicate neuroepithelial loops. Scale bars: 200uM

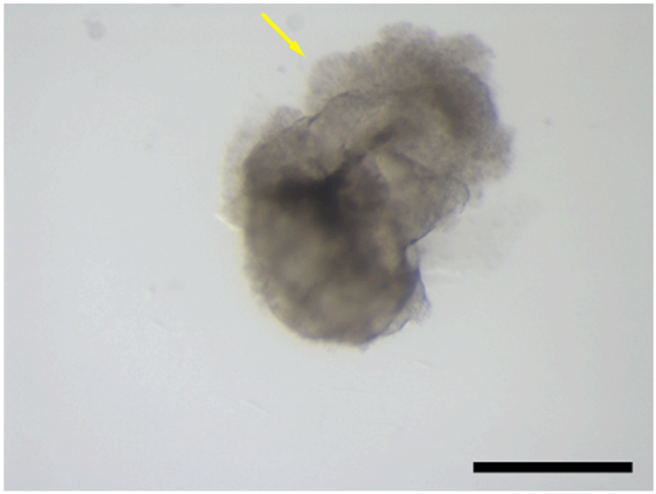


Fig. 3.12
Transdifferentiated RPE HH24 at 7 days can lose integrity in neuroepithelial loops (yellow arrow).
By 7 days in culture, some transdifferentiated RPE explants treated with bFGF appear to lose their structural integrity (yellow arrow) and can disintegrate without careful handling. It appears as though cells may undergo apoptosis after being in culture for a certain amount of time. This could be due to a lack of the presence of other crucial growth factors needed to support further retinal development, or perhaps an oxygen deficiency. Scale bar: 200uM.

phenotype following treatment with bFGF (Fig. 3.10, 3.11). Untreated aggregates retained their size and pigmentation throughout their time in culture (Fig. 3.10, 3.11). On all occasions (n=4), chick RPE of this stage displayed the ability to form non-pigmented, retina-like protrusions, hereafter referred to as 'neuroepithelial loops,' in response to bFGF (Fig. 3.10, 3.11). These were clearly visible after approximately 2 days in culture in the presence of bFGF. They appeared to be low in optical density with a lack of visible pigmentation akin the native retina. On most occasions (n=3), these neuroeptithelial loops protruded out from a region that remained pigmented, located at the centre of the aggregate. This region appeared to retain the characteristic RPE phenotype throughout the time in culture (Fig. 3.10). On more rare occasions (n=1), these pigmented regions were absent from cultures treated with bFGF, and the entirety of the RPE sheet appeared to have undergone transdifferentiation (Fig. 3.11). The resulting neuroepithelium was observed to increase in size during time in culture regardless of the presence of remaining regions of pigmentation (Fig. 3.10, 3.11), however, those aggregates which did contain regions of pigment appeared to exhibit an increase in size of the neuroepithelial loops, without a further loss in pigment at the centre of the aggregates (Fig. 3.10), which retained a similar size throughout the time in culture.

Interestingly, despite robust transdifferentiation observed in all explants taken at this developmental stage after by 7 days in culture, some of the neuroepithelial loops appeared to display a loss in structural integrity, and were more prone to disintegration without very careful handling (Fig. 3.12 yellow arrow). These neuroepithelia were also less transparent and smooth in appearance than other aggregates, which displayed a much more robust structure (Fig. 3.9, 3.10). It is possible that some areas of the neuroepithelium were undergoing apoptosis during their time in culture, potentially as a result of the fact that they were not adapted to the *in vitro* culture system.

Cross-sectional analysis of these aggregates reveals that there is indeed a significant loss of pigment in response to treatment with bFGF. This loss of pigment is correlated with the appearance of a large, multi-cellular, multi-layered neuroepithelium, largely made up of cells with nuclei which are positive for the neural progenitor markers Sox2 and Pax6

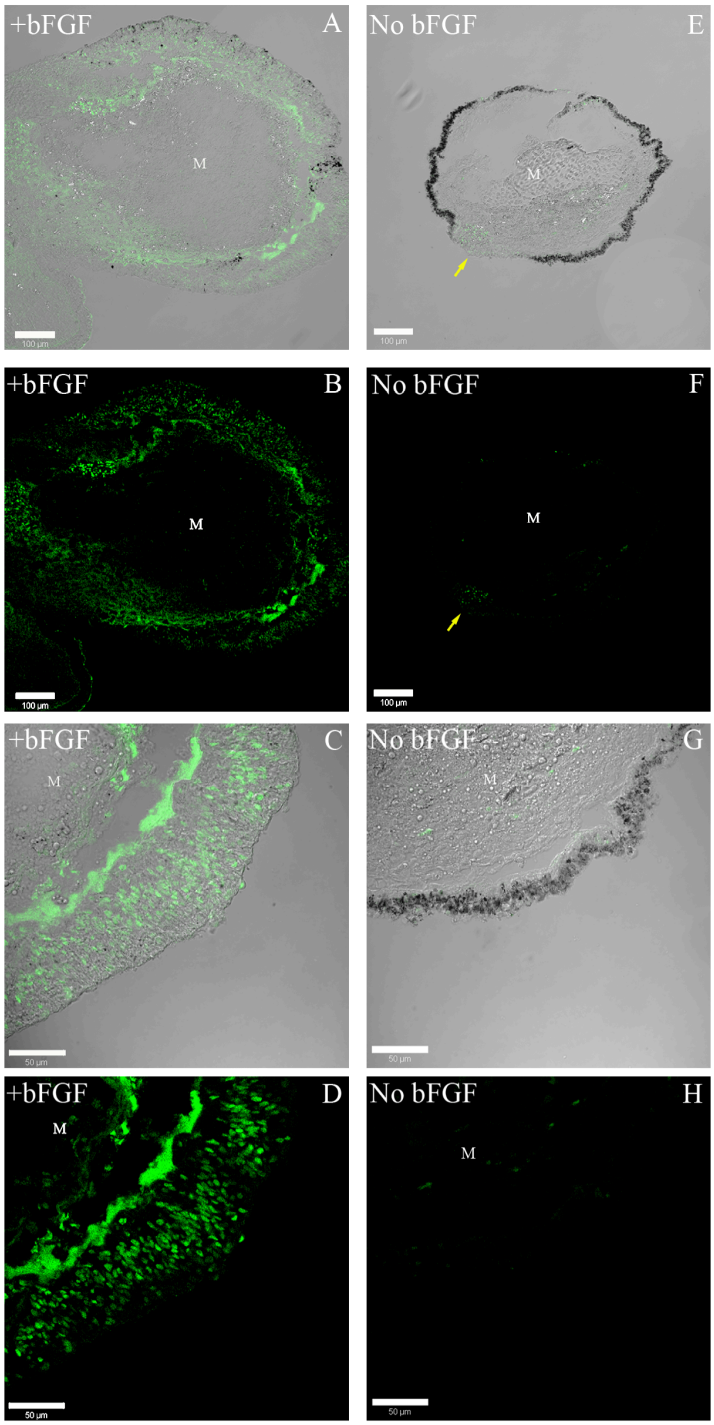


Fig. 3.13
Sox2 expression in Chick RPE HH24 +/- bFGF after 7 days in HESC medium. bFGF treated explants exhibited large, de-pigmented neuroepithelial loops which were found to contain a large number of Sox2 (green) positive nuclei (A-D). This phenotype is consistent with transdifferentiation of the RPE. The majority of untreated explants retained a characteristically pigmented, RPE monolayer phenotype which did not express Sox2 (G, H). However, one explant did exhibit a thinner, de-pigmented region with weak Sox2 expression in some of these cells (E, F yellow arrow), which resembles limited transdifferentiation. This most likely resulted from contamination of the RPE explants which expressed a low level of bFGF, in this particular explant. Fluoresence digitally enhanced. Scale bars: 100uM (A, B, E, F), 50uM (C, D, G, H). M = extraocular mesenchyme.

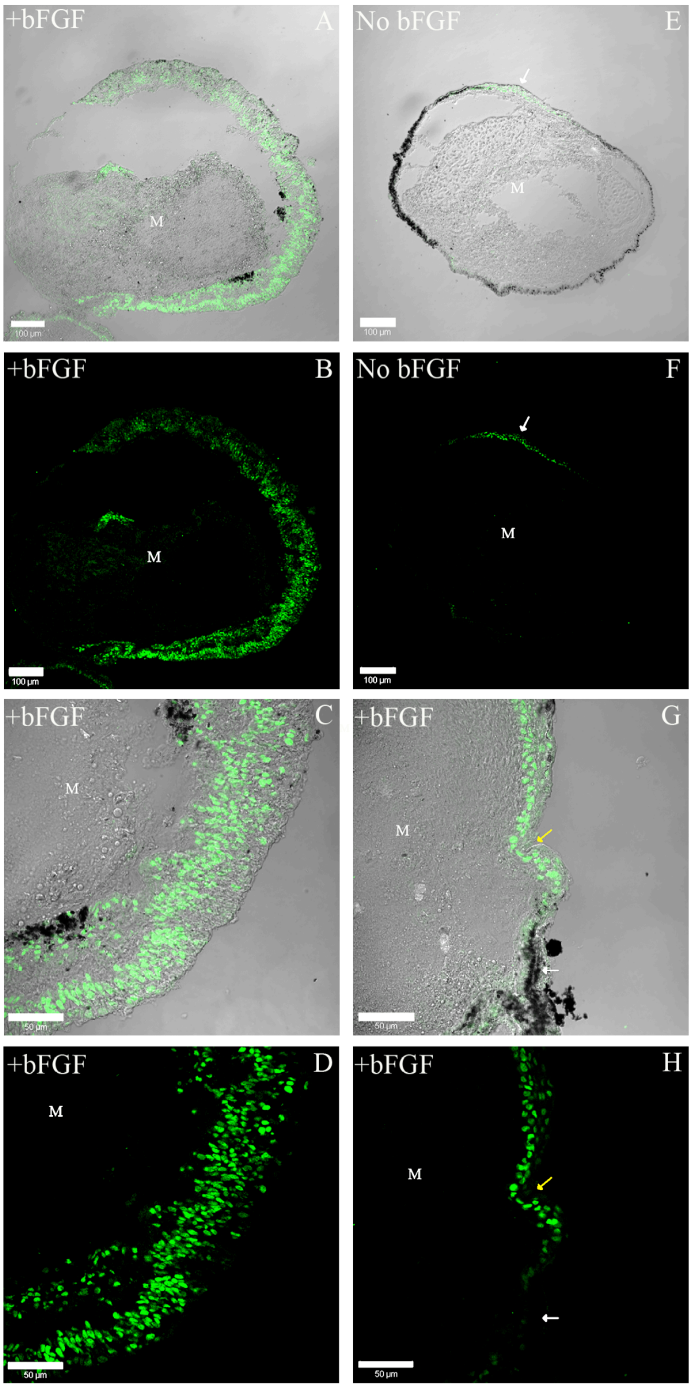


Fig. 3.14
Pax6 expression in Chick RPE HH24 +/-bFGF 7 days in HESC med.
bFGF treated RPE explants transdifferentiated to form Pax6 (green) positive, de-pigmented neuroepithelia (A-D) which was observed to be continuous with a single-layer of Pax6 negative, pigmented RPE cells (G, H, yellow arrows). Some transdifferentiated regions retained pigment granules throughout the neuroepithelium, reflecting the previous RPE specification (C). Untreated explants had down-regulated Pax6 after 7 days in culture, however, one explant did display a thin, multi-layered, region lacking in pigmentation, which did express Pax6. This would imply a small amount of transdifferentiation had taken place (E, F, white arrows), most likely as a result of retinal contamination releasing a small amount of bFGF in this explant. However, all other explants retained a pigmented RPE phenotype. Mesenchyme (M). Fluoresence digitally enhanced. Scale bars:100um (A, B, E, F), 50um (C, D, G, H).

(Figs. 13A- D, 3.14A-D respectively). Interestingly, the neuroepithelium still contains pigment granules which indicates its former specification as an RPE monolayer (Fig. 3.13A, C; 3.14A, C). Despite most of the RPE having undergone transdifferentiation, the regions which did not transdifferentiate, and maintained a pigmented RPE phenotype (Fig. Fig. 3.14G, H white arrow), were observed to be continuous with the neuroepithelium (Fig. Fig. 3.14G, H yellow arrow), implying that the neuroepithelium arose as a result of RPE transdifferentiation.

Interestingly, and perhaps unexpectedly, an aggregate which was not treated with bFGF, despite largely maintaining its heavily pigmented, RPE phenotype, did show a narrow region of de-pigmented RPE that exhibited some characteristics of transdifferentiation (Fig. 3.13E, F yellow arrows, 3.14E, F white arrows). This region, while not as thick as the neuroepithelium resulting from exogenous treatment with bFGF, did exhibit several layers of cells that weakly labeled for Pax6 (Fig. 3.13E, F white arrows), and Sox2 (Fig. 3.13E, F yellow arrows), expression. These regions were also continuous with the mostly pigmented, RPE monolayer at both ends of the neuroepithelium, indicating a shared developmental precursor for cells of both phenotypes (Fig. 3.13E, 3.14E). However, for the most part, untreated RPE retained its characteristic pigmented phenotype and remained negative for Pax6 (Fig. 3.14F) and Sox2 (Fig. 3.13F-H) expression.

3.2.4 Discussion:

Embryonic chicken RPE appears to be able to undergo transdifferentiation towards a neural retinal phenotype using this particular, human-cell optimized culture system. It is able to undertake this process in a similar manner to that of the chicken optimized culture system reported in the literature (Pittack et al., 1997, Pittack et al., 1991, Sakami et al., 2008). The non-adherent RPE sheets are able to form spherical aggregates irrespective of their treatment with exogenous factors, however, the mechanism for this is unclear. The de-pigmentation of the RPE aggregates following treatment with bFGF correlates with previous investigations

and would tend to suggest that a down-regulation of the RPE transcription factor Mitf has occurred (Mochii et al., 1998a, Shibahara et al., 2000, Baxter and Pavan, 2003). However, there is no direct evidence for this in this study. Mitf is known to be heavily implicated in control of the network of genes responsible for pigmentation, and is also critical in the proper differentiation and development of RPE cells (Mochii et al., 1998a). *In ovo* studies in the silver mutant quail (Mitf-/-) have shown that a loss in expression of the transcription factor pre-disposes the animal to micropthalmia and transdifferentiation of the RPE towards neural (Mochii et al., 1998b). It is thought that without the presence of Mitf, members of the FGF-family of growth factors, including FGF2 and FGF8, which are expressed by the over-lying surface ectoderm and retina, are able to act on the RPE and initiate transdifferentiation towards a neuroepithelial phenotype (Mochii et al., 1998a, Galy et al., 2002, Spence et al., 2007b, Vogel-Hopker et al., 2000, Nguyen and Arnheiter, 2000). The fact that Mitf is thought to negatively regulate the expression of Pax6 would suggest that, under normal circumstances, the presence of Mitf in the RPE is able to maintain low levels of Pax6 expression so that the RPE phenotype is maintained. If this fine-tuned balance is modified, for example: through application of higher concentrations of bFGF adjacent to the RPE, and a subsequent increase in Pax6 expression, Mitf expression, may be down-regulated. Additionally, if Mitf activity is lost via a mutation, then this results in a change in the RPE phenotype (Iwakiri et al., 2005, Manuel et al., 2008, Mochii et al., 1998a).

Neuroepithelia resulting from bFGF-induced transdifferentiation of the RPE cells appear to increase in size over time in culture, however, some regions of these explants retained their pigmentation and did not to transdifferentiate in response to bFGF. These regions tended to be located in this centre of the aggregates, and remained a consistent size throughout the time in culture. This would tend to suggest that the increase in size of the neuroepithelial loops result from the proliferation of the retinal progenitor cells produced by the initial transdifferentiation event, rather than further transdifferentiation of the remaining RPE cells. This is not unexpected given the fact that Pax6/Sox2 expressing neural progenitors are responsible for the proliferation and expansion of the retina during in normal

eye development (Li et al., 2012). The control of the levels of expression of these transcription factors regulates the balance between maintenance of a retinal stem cell-like progenitor state and the onset of differentiation into the various retinal cell types, including photoreceptors (Oron-Karni et al., 2008). There could perhaps be a concern that the neuroepithelia in bFGF treated cultures result from contaminant retinal progenitors in the original preparations, which subsequently proliferate in response to a bFGF stimulus (a growth factor heavily implicated in retinal development (Pittack et al., 1997, Martinez-Morales et al., 2005)). This is of particular relevance given that some regions of pigmented RPE remain unchanged during culture. However, the contamination is very unlikely owing to the fact that some aggregates do not retain pigmented regions, and therefore, in order to produce the observed neuroepithelia, all of the pigmented RPE must have undergone transdifferentiation towards a neuronal phenotype, rather than contaminant progenitors having proliferated. The fact that no RPE remain and only neuroretinal cells are present following treatment with bFGF would strongly suggest that the RPE cells are the source of the novel retina.

It is unclear why so many of the bFGF treated RPE explants retain some pigmented, non-transdifferentiated regions. It is possible that portions of these explants have already lost the capacity to respond to bFGF, in a similar manner to the rest of the RPE as development progresses. Should this be the case, the mechanism responsible would most likely be involved in the loss in capacity for transdifferentiation at later developmental stages. If RPE is to be used as a source of retinal cells, then understanding how to induce transdifferentiation in later stage RPE will be very important. However, other factors may cause a portion of the RPE not to transdifferentiate. The fact that in most cases these regions appear in the centre of the aggregate could suggest that perhaps the exogenously-added bFGF is unable to contact these cells and trigger transdifferentiation.

There may also be other micro-environmental effects that prevent these regions from transdifferentiating. For example, the RPE themselves may be releasing autocrine signals which augment the RPE phenotype, and inhibit the effect of the exogenous bFGF. Developing

RPE cells and adjacent mesenchymal tissue (present in many explants) have been shown to express TGFβ-family growth factors, such as activin, which have been reported to display an inhibitory effect on bFGF-induced transdifferentiation (Sakami et al., 2008). It is thought that this occurs via an augmentation of the RPE phenotype (Fuhrmann et al., 2000b). Inhibition of transdifferentiation could result as a consequence of certain regions experiencing higher concentrations of these signals, and therefore be more resistant to transdifferentiation. Similarly, there may be an inhibitory signal being released from the over-lying, novel retina, which protects the remaining RPE from transdifferentiating. Micro-environmental fluctuations in the concentrations of such factors, should they exist, could account for the variability in whether or not aggregates do, or do not, retain some pigmented regions. However, given the size of the aggregates, it seems unlikely that the concentrations of such factors would be high enough to inhibit the effects of a high dose of exogenous bFGF.

Another factor may simply be the presence of physical restraints, which effect the ability of these pigmented regions to undergo transdifferentiation, given that the neuroepithlial loops are relatively large and may need room to develop properly. This again seems unlikely given that RPE cells are observed to promptly lose pigmentation before these neuroepithelia have formed, and therefore it is unlikely that transdifferentiating RPE require particular spatial requirements, at least in the initial stages of the phenomenon. Some investigations have reported that physical constraints, including the type of extracellular matrices, and their physical properties, can influence the ability of RPE to undergo transdifferentiation, so this is certainly possible (Opas and Dziak, 1994b).

Some of the aggregates appeared to lose their integrity by the time they have been in culture for 7 days, however, the reason for this is unclear. It is possible that a medium osmotically optimized for human cells, could cause some of the cells to lyse, however, this seems unlikely given that the same RPE appears to survive well in the same basal media, unless retinal cells are somehow more sensitive to osmotic changes than RPE cells. It is more likely that the retina requires a number of supportive factors that are not necessarily present in this *in vitro* culture system. For example, the fact that these transdifferentiated retinas develop

in a more disorganised fashion than that of the native retina could mean that the cells comprising the neuroepithelium are not receiving the correct, spatio-temporal, developmental signals. This may cause the transdifferentiated retina to undergo apoptosis at an earlier stage than expected. The loss in integrity could also be attributed to the lack of support, both physical, and chemical, for these neuroepithelia in non-adherent cultures. Particularly given that the retina is unable to develop properly without the presence of an underlying RPE (Raymond and Jackson, 1995). Perhaps the most obvious reason for cell death in the developing, transdifferentiated retina is oxygen deprivation. In normal development, the developing retina is vascularized from very early in development, which means that it has a good supply of oxygen to support the respiratory demands of rapid growth and differentiation occurring in developing retina. Despite the fact that these transdifferentiated retinas are relatively thin, and one might expect that this would allow sufficient oxygen transfer via diffusion in culture, this is unlikely to compensate for the level of oxygenation a normal retina would receive via the extensive array of vasculature. A recent study that reported the differentiation of human embryonic stem cell aggregates into whole eye cups supports this hypothesis. Once the presumptive retina in these aggregates had formed, the investigators increased the oxygen level from the normal atmospheric level (as in this investigation) to approximately 40% oxygen (Nakano et al., 2012). It is likely that this increase in the oxygen diffusion gradient would allow for better aeration of the neuro-epithelium. This study has shown that the resulting retinas can be sustained for at least number of months.

The presence of a small number of pigment granules in the transdifferentiated neuroepithleium indicates its former specification as an RPE cell. It is reasonable to suggest that given the intense level of pigmentation of the RPE, and therefore the high number of granules to be present, it is perhaps unsurprising that some pigment granules remain in the re-specified cells. One would expect that given more time in culture that these would eventually disappear as the neuroepithelium develops.

The neuroepithelium was positive for neuroprogenitor markers Pax6 and Sox2 highlighting that it is still early in its development. Both of these markers are known to be

down-regulated in most differentiating cell types of the retina as it progresses through its development. Despite the apparent necessity for Pax6 expression in the developing RPE (Baumer et al., 2003, Bharti et al., 2012) at early stages of development, over-expression of Pax6 in RPE cells causes them to lose their RPE phenotype via induction of transdifferentiation (Azuma et al., 2005a). Similarly, a down-regulation of Pax6 in the retina is required for the differentiation of photoreceptors (Oron-Karni et al., 2008), and, in conjunction with Sox2 expression, is known to regulate a careful balance between maintenance of a stem cell-like state and differentiation/neurogenesis (Matsushima et al., 2011). The regulation and function of Pax6 and Sox2 in the eye is very complex, and it is clear that they interact in the process of development. For example, it has been shown that they are able to act synergistically in order to enhance Pax6 expression in lens development via the activation of a head surface ectoderm-specific, enhancer element in the Pax6 gene (Cvekl et al., 2004, Ashery-Padan et al., 2000, Aota et al., 2003).

It is widely accepted that an increase in Pax6 expression is known to initiate the process of RPE to neuroretinal transdifferentiation as previously discussed. In contrast, little investigation into the role of Sox2 has been reported. However, it has been shown that an over-expression of Sox2 in embryonic chicken retinas (E2.5-E3) is able to induce a level of transdifferentiation of the host RPE cells (by E8) (Ma et al., 2009). This involves the de-pigmentation of regions of the RPE monolayer, a significant down-regulation of RPE marker pigment epithelium-derived factor (PEDF), and an up-regulation of early ganglion cell markers RA4 and neurofilament-associated protein (as labeled with 3A10 antibody). However, despite this apparent transdifferentiation taking place, the de-pigmented regions were found to be negative for ganglion cell markers Brn3a or Islet-1, in addition to general neuronal marker Map2, Pax6, or photoreceptor marker visinin. This would suggest that Sox2 may be somewhat involved in the early induction of transdifferentiation but requires other factors the initiate compete differentiation of a neuroepithlium from RPE cells. It may be that the reported lack of Pax6 expression would account for this missing component in the induction pathway.

In another experiment, embryonic chicken RPE (E6) was dissociated and grown in culture until confluence. Dissociated RPE was seen to lose its characteristic cobblestone morphology and pigmentation, in keeping with the de-differentiation expected from dissociated RPE. These cells were able to proliferate and regain a normal RPE phenotype upon confluence. However, over-expression of Sox2 in these cultures prevented the cultures from re-differentiating. In addition, a large number of $RA4^+$ and $3A10^+$ cells were present, which in some cases displayed a neuronal-like morphology with out-growth of neuronal processes. This apparent neuronal differentiation was accompanied with a down-regulation in the transcription of important RPE genes, Mitf, Otx2 and MMP115. In this system, over-expression of Sox2 in the RPE also resulted in an induction of Pax6 in RPE cells, which could imply that Sox2 regulates a transcription network in RPE which can up-regulate Pax6 transcription, potentially via a similar feedback loop as in the lens as previously discussed. Dissociated cultures of chick RPE treated with bFGF elicited a similar response, however, many of the $RA4^+$ cells failed to display a neuronal morphology, suggesting that bFGF may be partially utilized in the Sox2 response.

Interestingly, it was shown that over-expression of Sox2 in whole retinas elicited an up-regulation in the expression on Pax6 in the retina. However, this change was not observed within the RPE. It is possible that this up-regulation in bFGF expression in the retina adjacent to the RPE could be responsible for the induction of transdifferentiation, however, one would expect bFGF induced transdifferentiation to up-regulate Pax6, which was reported to be absent. In contrast, RPE cell cultures with over-expression of Sox2 exhibited a more than 2-fold increase in bFGF expression. This could possibly account for the up-regulation in Pax6 expression in cultured RPE with Sox2 over-expression. Interestingly, it has also been shown that in a similar cell culture system, treatment with bFGF leads to an up-regulation of Sox2 in RPE cells, much like that observed in this study (Sakami et al., 2008, Ma et al., 2009). This would suggest that bFGF and Sox2 are able to act synergistically in order to augment one another's expression during development. Other studies have demonstrated that importance of bFGF in maintenance of a stem cell-like state via an up-regulation/maintenance of Sox2 (Xu

et al., 2005, Wu et al., 2012, Furue et al., 2008). Similarly, it is common practice for bFGF to be used in the culture of embryonic/iPS-stem cells in order to maintain a pluripotent state, as well as the fact that Sox2 is one of the components that is regularly over-expressed in fibroblasts to influence cells to become iPS cells (Yamanaka, 2008, Liu et al., 2011). It is possible that the early induction of Sox2 expression in RPE cells confers a stem cell-like state, which allows them to undergo proliferation and differentiation into cells of a different phenotype.

Spatio-temporal, genetic ablation of Sox2 in the developing mouse eye completely removed to potential for neurogenesis (Matsushima et al., 2011). Cells eventually underwent a fate change to a non-neurogenic, ciliary epithelial phenotype. This specification was interestingly accompanied by a significant increase if Pax6 expression which would again suggest that the control of Sox2 and Pax6 transcription are closely linked, and that in neural progenitors, Sox2 is somehow responsible for moderating Pax6 expression. The fact that in this instance Sox2 appears to negatively regulate Pax6 expression in contrast with Sox2 over-expression studies is curious and certainly points to a complex relationship between the two transcription factors. It may be that this relationship is different across different species. However, what is clear is that Sox2 is critical for the neural competence of the developing eye, which might explain the up-regulation of a select group of neuronal markers following over-expression in the RPE.

In the current study, limited transdifferentiation was observed in one, untreated explant. This transdifferentiation does not appear to be as robust as that of bFGF-treated samples, as displayed by the fact that the de-pigmented region is much smaller and thinner than in explants with exogenous bFGF. In addition, most of the RPE in this explant did appear to retain its characteristic pigmented phenotype. It is possible that this response was elicited by the standard culture medium, or possibly by a response to the mechanical dissection of the RPE from the eye-cup. A similar phenomenon has been observed in the newt RPE which up-regulates Pax6 expression when removed from the adjacent choroid independently of bFGF, however, no change in Sox2 was reported (Kuriyama et al., 2009a).

This report demonstrated that bFGF treatment was merely required for maintenance of Pax6 expression and not its induction. However, both of these hypotheses seem unlikely given the fact that this minimal transdifferentiation was an isolated result. Other controls did not display this phenomenon. It is therefore more likely that some contaminating tissue was present that was secreting growth factors, possibly a low concentration of bFGF itself, and thus inducing minimal transdifferentiation. Given that mesenchymal tissue which is still associated with the basal surface of the RPE is known to augment the RPE phenotype, rather than initiate a departure from it (Fuhrmann et al., 2000b), it is most likely that some small amount of FGF-expressing retinal contaminant was present (Consigli et al., 1993, Hyuga et al., 1993, Zhao et al., 2001, Coulombre, 1981, Coulombre and Coulombre, 1965, Coulombre and Coulombre, 1970). Additionally, other investigations employing similar culture methods, where the mesenchymal tissue remains attached to the RPE, did not report the same phenomenon. In future, it will be necessary to ensure the purity of RPE cell explants used in this investigation, as well as to examine whether the dissection process itself has any inductive effects of the RPE (see later in this chapter).

3.3 Further characterisation of transdifferentiated chick RPE

3.3.1 Introduction:

In order to better understand the development of the retina which results from bFGF-induced transdifferentiation of embryonic chick RPE cells in culture, it was necessary to further analyse the expression of a number of other markers associated with the development of a neuronal phenotype, in addition to specific retinal phenotypes.

3.3.2 Materials & Methods:

Methods are described in materials and methods for experiment 3.2.

3.3.3 Results:

A summary of retinal marker expression in transdifferentiated chick RPE explants +/- bFGF is displayed in table 3.2.

3.3.3.1 α-acetylated-tubulin:

Both bFGF treated, and untreated chick HH24 RPE explants were observed to express the early neuronal lineage marker α-acetylated-tubulin (Fig. 3.15A, B; C, D respectively) following 7 days in non-adherent culture. The expression pattern of α-acetylated-tubulin in transdifferentiated RPE, +bFGF, was observed to be cytopasmic, column-like, neuronal patterning expected of a developing neuroepithelium (Fig. 3.15A, B). Unexpectedly, non-treated, pigmented RPE that retained an epithelial phenotype was also shown to exhibit a similar cytoplasmic pattern, albeit without the neuronal morphology (Fig. 3.15C, D). Therefore, the expression of this general, early neuronal marker was not dependent upon treatment with bFGF, and as a consequence, the onset of transdifferentiation.

3.3.3.2 HuD:

In addition to the expression of retinal progenitor markers, transdifferentiated retina also exhibited the expression of differentiated markers of specific retinal phenotypes. This included the expression of ganglion cell and amacrine marker, HuD, which was expressed in

Marker	+bFGF Transdifferentiated retina	-bFGF RPE phenotype
Sox2	+++	-
Pax6	+++	-
alpha-acetylated-tubulin	+++	+++
HuD	++	-
Islet1	++	-
Otx2	++	+++
Crx	++	+++
Rhodopsin	+	-

Table 3.2
A summary of the expression of retinal markers in chick RPE HH24 explants treated in standard, non-adherent, transdifferentiation culture conditions, +/- bFGF for 7 days.
Robust positive labeling in most transdifferentiated cells (+++). Many cells observed to express marker (++). Very few cells express marker (+). Negative labeling (-).

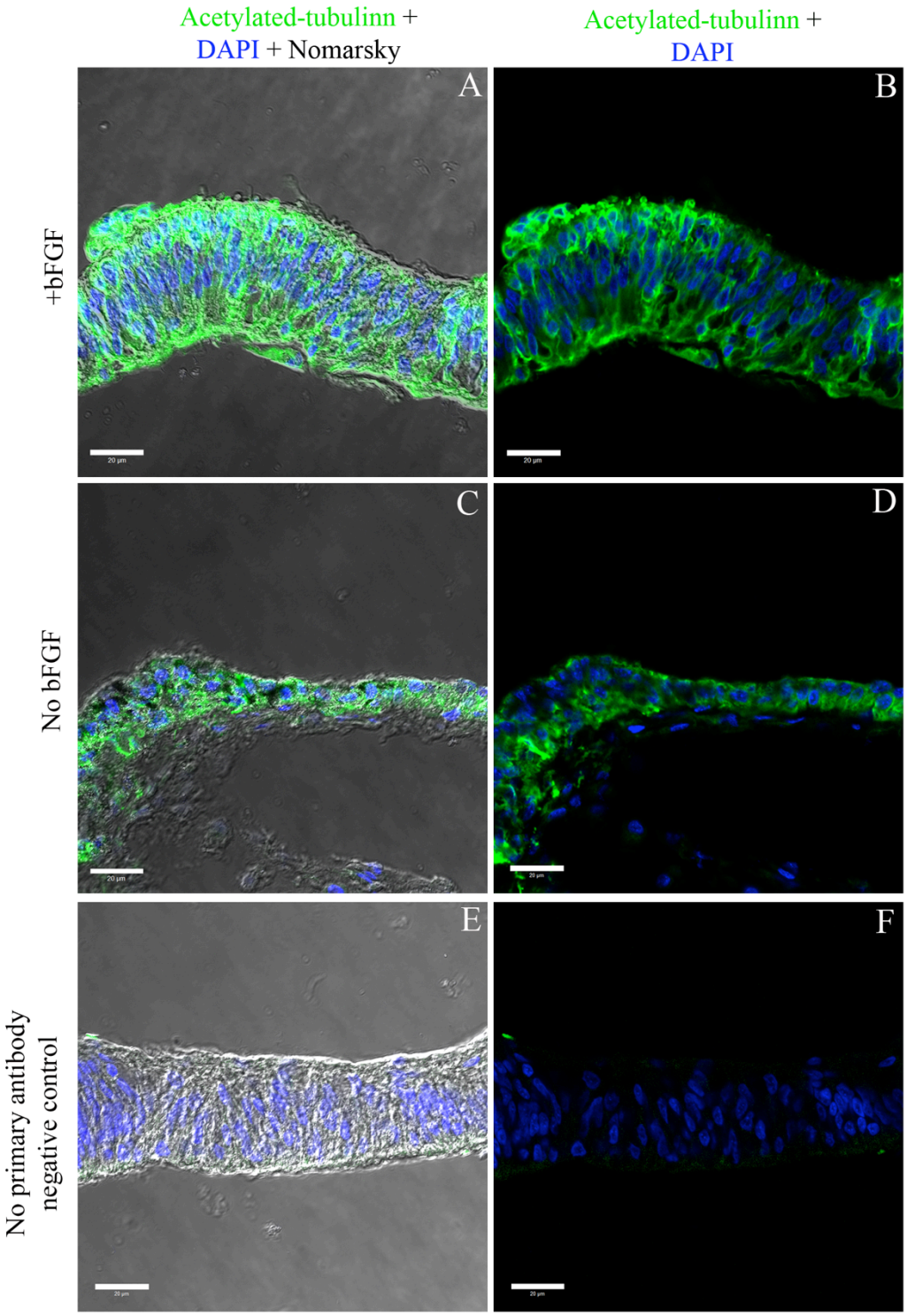


Fig. 3.15
alpha-acetylated-tubulin expression in Chick RPE HH24 +/- bFGF for 7 days in HESC medium.
Transdifferentiated RPE treated with bFGF expressed alpha-acetylated-tubulin (green) throughout the multi-layered (DAPI, blue) neuroepithelium (A, B). The pattern of expression indcates a neuronal, columnar, phenotype characteristic of native retinal cells (A, B). However, untransdifferentiated, untreated, RPE explants which retained the pigmented RPE phenotype were also observed to express alpha-acetylated-tubulin. No signal was detected in no primary antibody negative controls (E, F). Scale bars: 20um.

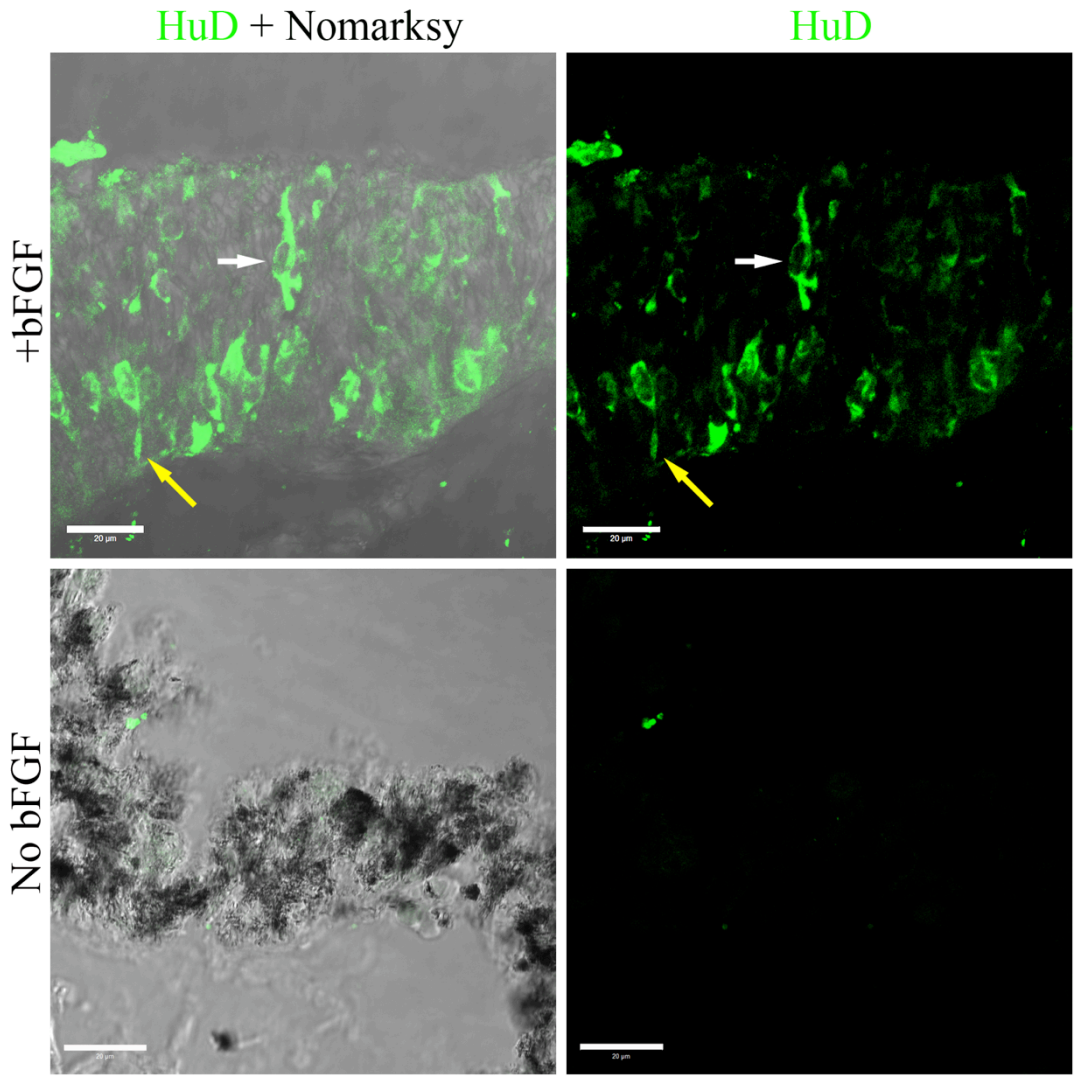


Fig. 3.16
HuD expression in Chick RPE +/- bFGF for 7 days in HESC medium.
Transdifferentiated RPE explants exhibited robust HuD (green) expression (A, B) in a number of cells. The majority of cells expressing HuD were localised to the basal surface of the novel retina (A, B, yellow arrows), indicating an inverted phenotype. Some HuD positive cells were also observed within the middle of the neuroepithelium, however, these were generally fewer in number than those at the basal surface (A, B, white arrows). Untreated negative control explants did not express HuD (C, D). Fluoresence digitally enhanced. Scale bars: 20um.

the bFGF – induced, transdifferentiated retina only (Fig. 3.16). No HuD expression was observed in untreated, negative control explants, indicating that HuD is a good marker of transdifferentiation (Fig. 3.16). HuD expression was labeled within the cytoplasm of a number of cells within the non-pigmented, retinal neuroepithelium, the majority of which were localized to the basal surface of the neuroepithelium (Fig. 3.16 yellow arrows), the opposite of that observed in developing native retina (Fig. 3.1i, 3.1ii), which implies that the phenotype of the transdifferentiated retina in inverted with respect to that of native retina. However, a few cells were observed to express HuD within the middle of the neuroepithelium (Fig. 3.16 white arrows), which may be ganglion cells in the process of migrating towards the basal surface where the presumptive ganglion cell layer is developing, or they may be differentiating amacrine cells in the middle of the developing retina. Interestingly, some areas of the transdifferentiated neuroepithelium appeared to be less structurally organized, in particular the HuD expression which was observed throughout the breadth of the novel retina, rather than being localized to particular regions (data not shown). It is possible that these areas have not been sectioned perpendicularly to the run of the retina, given the erratic structure of the cultured transdifferentiated RPE explants. Despite this, the majority of the HuD expression observed in transdifferentiated retina is characteristic of normal, inverted, retinal development.

3.3.3.3 Islet1:

Similarly, another ganglion cell marker, Islet-1, was observed to be expressed in the nuclei of a number of cells along the basal surface of the transdifferentiated retina (Fig. 3.17 yellow arrows). Other cells in the retina were negative for Islet-1 expression, which is consistent with its expression in developing retinal ganglion cells only. No Islet-1 expression was visible in the untreated, negative control RPE explant, which retained its heavy pigmentation (Fig. 3.17).

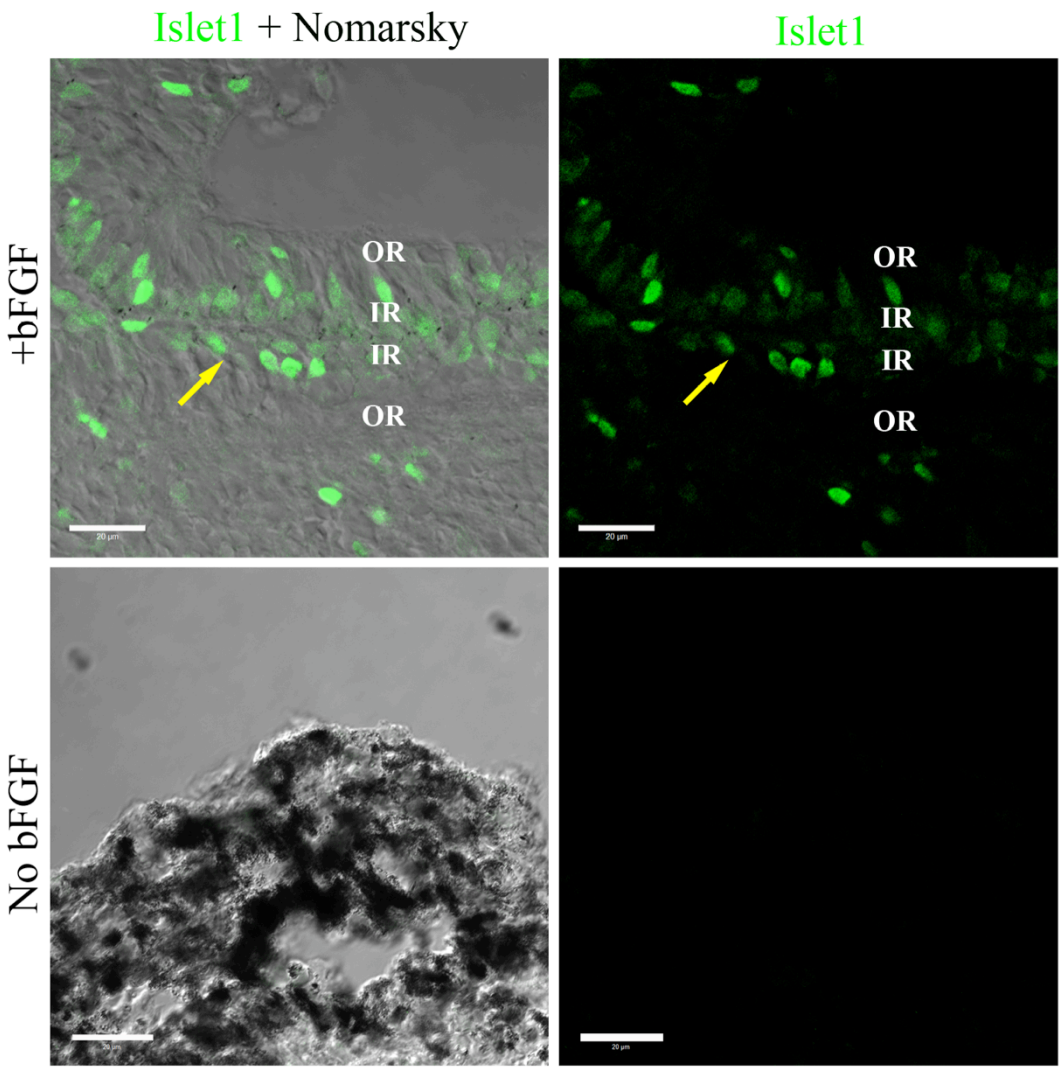


Fig. 3.17
Islet-1 expression in Chick RPE HH24 +/- bFGF for 7 days in HESC medium. Transdifferentiated RPE explants robustly expressed Islet-1 (green) in the nuclei of a layer of cells which were localised to the basal surface of the neuroepithelium (A, B, yellow arrows). The localisation of these cells suggests that Islet-1 labels differentiating ganglion cells in the transdifferentiated retina. No Islet-1 expression was observed in un-treated controls (C, D). Outer retina (OR), Inner retina (IR). Scale bars: 20um.

3.3.3.4 Otx2:

The transcription factor Otx2 was expressed in both bFGF treated, and untreated RPE explants (Fig. 3.18). Transdifferentiated retina was observed to express Otx2 in a number of non-pigmented, neuro-retinal cells (Fig. 3.18A, B). Otx2 was expressed within the nuclei of these cells, which appeared to be generally localized to the basal surface of the novel retina, where the presumptive photoreceptor layer is likely to develop, once again indicating an inverted, retinal phenotype (Fig. 3.18A, B). Additionally, many cells throughout the middle of the neuroepithelium were also observed to express Otx2 (Fig. 3.18A, B). As one would expect, untreated RPE explants that retained their characteristic pigmented phenotype, also retained the expression of Otx2 in all cells (Fig. 3.18C, D).

3.3.3.5 CRX:

As expected, cone-rod homeobox transcription factor, CRX was robustly expressed in a number of nuclei situated at the outer edge of the neuroepithleium (Fig. 3.19A, B). Given the apparent inverted nature of the transdifferentiated neuroepithelium, this is where the developing photoreceptor layer normally be found. In addition, a small proportion of CRX-positive nuclei were found scattered towards the inner edge (Fig. 3.19A, B yellow arrows) in a pattern distinct from the densely labeled layer of cells presumed to be developing photoreceptors (Fig. 3.18A, B). Perhaps unexpectedly, untreated RPE cells that retained their pigmented phenotype also appeared to express CRX in all nuclei of the explant (Fig. 3.19C, D). This positive signal did not appear to be as a result of non-specific labeling of the antibody.

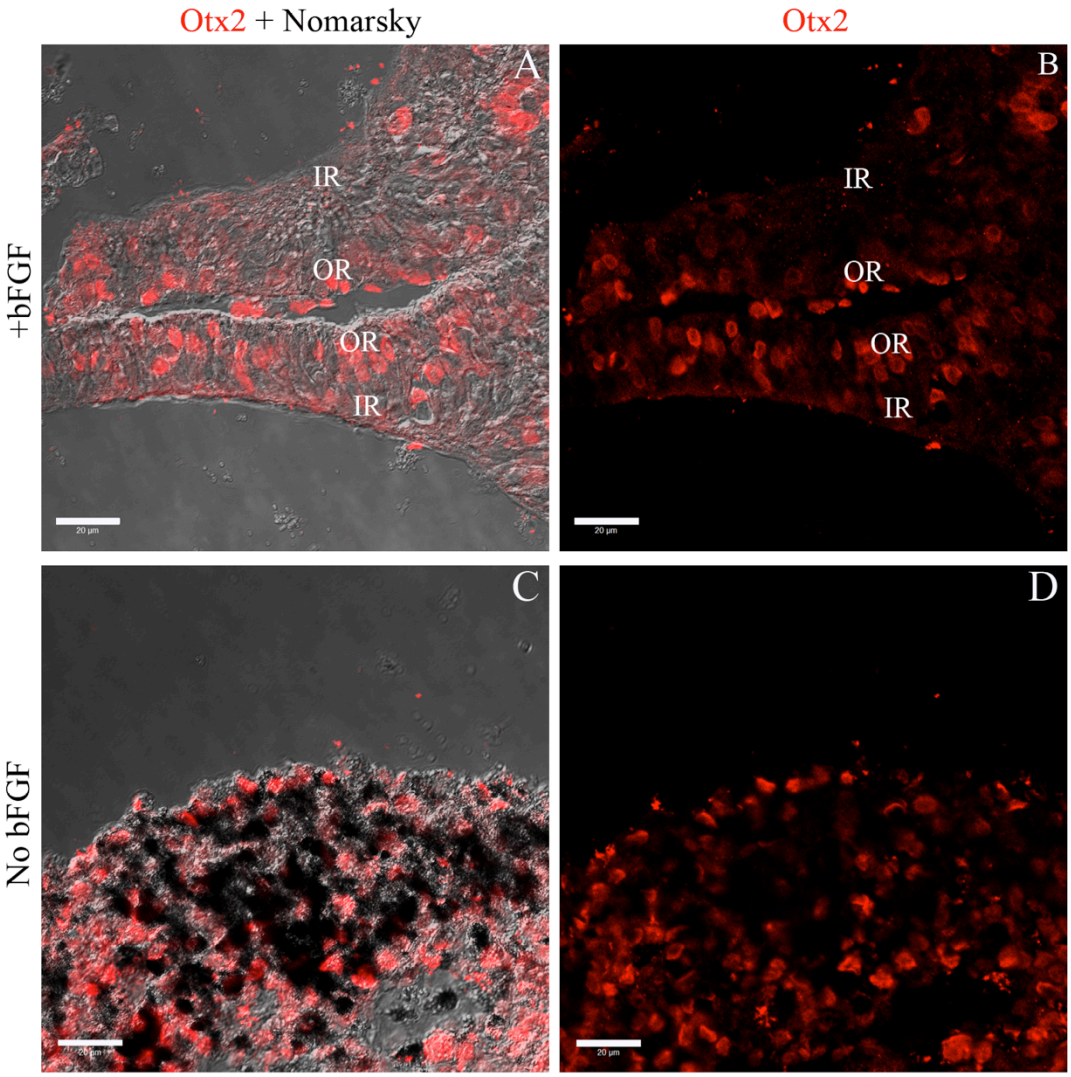
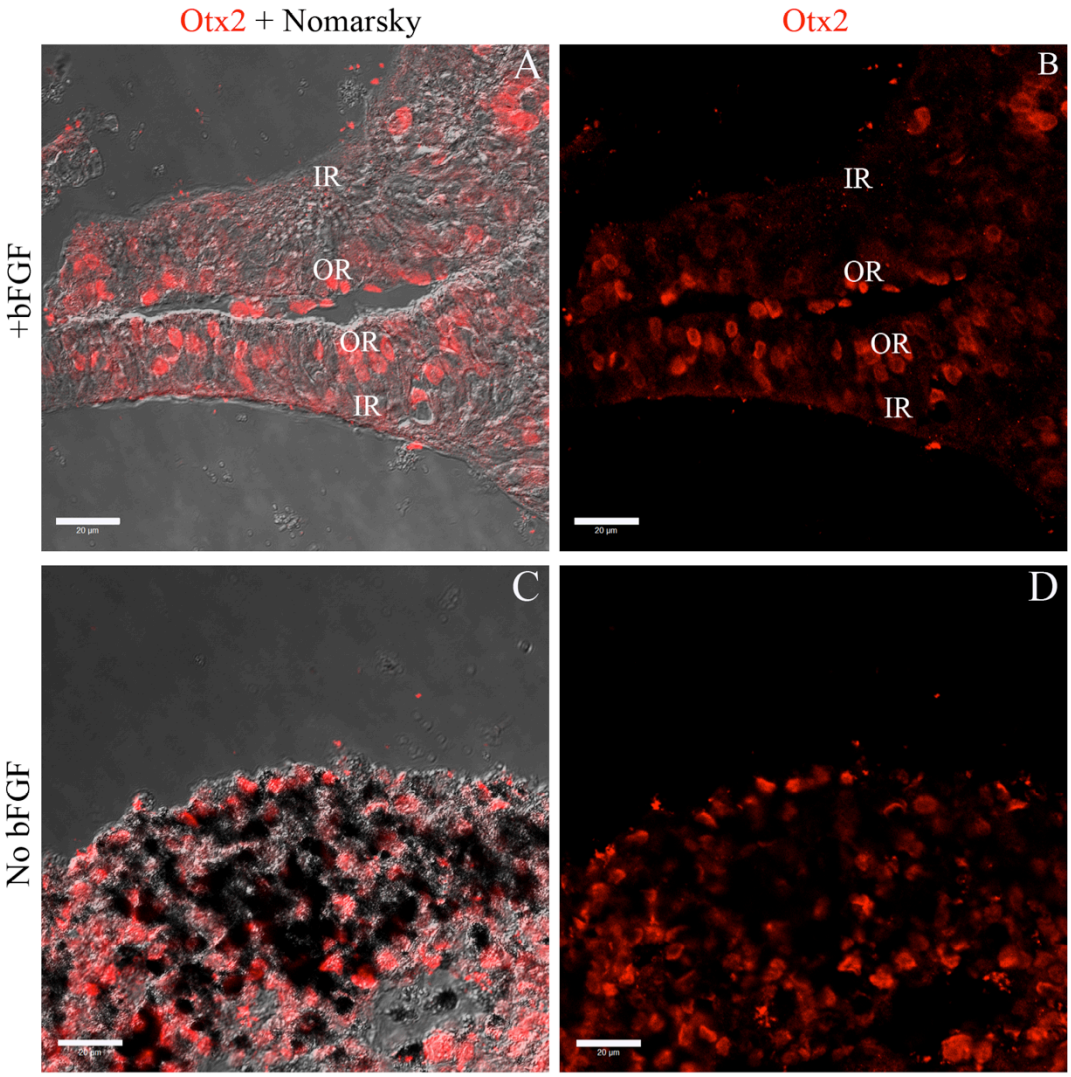


Fig. 3.18
Otx2 expression in Chick RPE +/- bFGF after 7 days in HESC medium.
Transdifferentiated RPE +bFGF exhibited robust expression of Otx2 (red) throughout the neuroepithelium, with a possible clustering of many positive cells towards the outer layer of the neuroepithelium, where developing photoreceptors are likely to develop (A, B). Otx2 expression was also robustly expressed in all pigmented RPE cells in untreated explants, which retained an RPE phenotype (C, D). Inner retina (IR), Outer retina (OR). Scale bars: 20um.



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3.3.3.6 Rhodopsin:

Encouragingly, in addition to the expression of several retinal cell specific markers: HuD and Islet-1, in transdifferentiated retina, the transdifferentiated neuroepithelium was shown to express a limited amount of rhodopsin, the membrane-bound, photosensitive protein responsible for hyper-polarization of rod photoreceptors in response to light, in a number of different explants (Fig. 3.20A, B, E, F). However rhodopsin was absent from all explants that were not treated with bFGF (Fig. 3.20C, D). This rhodopsin expression was robust but limited to a small number of cells located at the outer edge of the neuroepithelium, accordant with the apparent inverted nature of the transdifferentiated retina (Fig. 3.20A, B white boxes). The protein was localized to the membrane of these cells as expected, and the morphology of the cells was characteristic of immature rod photoreceptors (Fig. 3.20A, B, E). Rhodopsin positive cells also displayed neurite-like processes extending outward from the retinal side of the cells into the neuroepithelium (Fig. 3.20E yellow arrows). The specificity of the rhodopsin antibody was confirmed via labeling of adult, chicken, retinal tissue, and positively labeled cells were confined to the photoreceptor layer (ONL) of adult retina, as expected (Fig. 3.20G). Confirmation of the expression of rhodopsin in explants was confirmed via RT-PCR and was found to be weakly expressed in RPE explants transdifferentiated in response to bFGF only (Fig. 3.20F). Non-treated explants did not exhibit rhodopsin expression by RT-PCR, which was consistent with immunohistochemical observations (Fig. 3.20F). Interestingly, not all explants which transdifferentiated in response to bFGF appeared to contain rhodopsin-labeled cells. Neither did all explants contain rhodopsin transcripts (approximately 50%, n=4).

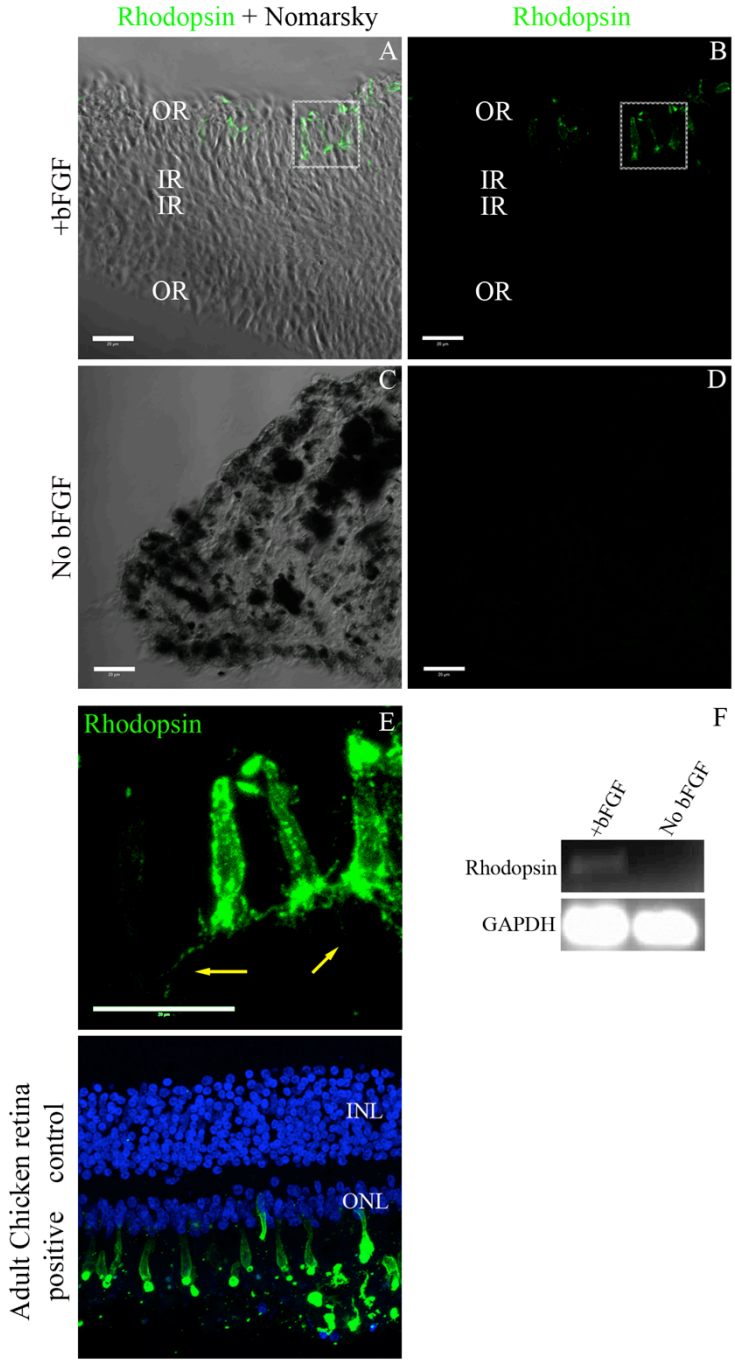


Fig. 3.20

Rhodopsin expression in chick RPE +/- bFGF at 7 days in HESC med.

Transdifferentiaited RPE was observed to express rhodopsin in a few cells in some explants (A, B, E), whereas, untreated negative controls were negative for rhodopsin expression (C, D, G). Rhodopsin was localised to the outer surface of the transdifferentiated neuroepithelium (A, B), indicating an inverted retinal phenotype. (E) High mag. images of rhodopsin expression in A, B, white boxes revealed that positive cells displayed an immature photoreceptor phenotype characteristic of rods. Additionally, neuronal processes extended out from these cells (yellow arrows) as expected of retinal neurons. (F) Rhodopsin transcripts in transdifferentiated RPE explants were confirmed by RT-PCR. The positive band was weak as a result of only a few positive cells being found in each transdifferentiated explant (F). Untreated explants did not express rhodopsin. (G) Positive control adult chick retina was labeled for rhodopsin in the outer nuclear layer as expected. Inner retina (IR), Outer retina (OR), ONL (outer nuclear layer), INL (inner nuclear layer). Scale bars: 20uM.

3.3.4 Discussion:

In addition to the expression of retinal progenitor markers Pax6 and Sox2, the transdifferentiated neural retina was also observed to express several other markers associated with native retinal development, as well as several differentiated markers of specific retinal cell types. This is encouraging for the on-going development of the neuroepithelium *in vitro*, following transdifferentiation in response to bFGF.

3.3.4.1 α-acetylated-tubulin:

It has been reported that pan-retinal-neuronal markers such as α-acetylated-tubulin are expressed at an early stage in transdifferentiating newt RPE cells (Susaki and Chiba, 2007, Mitsuda et al., 2005, Ikegami et al., 2002). Interestingly, α-acetylated-tubulin expression was observed in both bFGF-treated and non-treated RPE cells in this culture system. One would expect that transdifferentiated neuroepithelium would be strongly-labelled for this neural marker, as observed, however, it is more surprising that RPE which maintains its characteristic phenotype was also observed to express this marker. It may be that the non-adherent culture system itself is able to induce a general, neuronal specification in the RPE cells, especially given that this marker is not observed in adherent culture of newt RPE cells (Mitsuda et al., 2005). However, this seems unlikely given the lack of other neural retina markers expressed in other untreated explants. It may also be that the expression of this marker in RPE cells reflects their neuro-ectodermal lineage in development (Galy et al., 2002, Klimanskaya, 2006, Nishina et al., 1999, Zhao et al., 2001). This primary antibody may have undergone non-specific binding to the explants, however, given the neuronal expression pattern of the marker as labelled with this antibody in the neuroepithelium, the pattern characteristic off neuronal cells, this again seems unlikely.

3.3.4.2 HuD & Islet1:

HuD is an RNA-binding protein expressed early in neuronal development and has also been implicated in neuronal plasticity (Perrone-Bizzozero and Bolognani, 2002, Szabo et al., 1991, Sakami et al., 2008). In the retina, it is generally associated with developing amacrine and ganglion cells (Sakami et al., 2008). Similarly, LIM-homeodomain containing Islet class factor-1 (Islet-1) is also critical for early neurogenesis (Varela-Echavarria et al., 1996).

Islet-1 null mutants die before the onset of retinal neurogenesis, however, tissue-specific knockdown of the protein subsequently results in a significant loss of several retinal cell types, including bioploar, amacrine and ganglion cells, which is reflected in a considerable loss of retinal function and thus vision (Elshatory et al., 2007). It has been demonstrated that this protein is required for general bipolar cell specification as well as ganglion cell differentiation (Elshatory et al., 2007).

Both HuD and Islet-1 expression have been reported as a result of RPE transdifferentiation in the embryonic chicken model and mouse (Sakami et al., 2008). The fact that HuD and Islet-1 expression were observed in the transdifferentiated neuroepithelium in response to bFGF-treatment would suggest that transdifferentiation of the RPE can yield cells which are specifically differentiated towards ganglion cell/inner nuclear layer lineages in a manner similar to that of the native developing retina. Islet-1 expression in the neuroepithelium of transdifferentiated mouse RPE was not observed following bFGF-treatment alone, but also required an additional pharmacological blockage of TGFβ/activin signaling to initiate Islet-1 expression (Sakami et al., 2008). In this embryonic chicken culture system, however, both markers were robustly expressed in cells at the inner edge of the transdifferentiated neuroepithelium, as opposed to the outer edge as observed in the native retina. This would suggest that the transdifferentiated retina in inverted with respect to the native retina, as has been reported previously (Sakami et al., 2008, Opas and Dziak, 1994b, Azuma et al., 2005a, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and

Hollenberg, 1993, Pittack et al., 1997). However, in some areas this polarization was not as clear as others, which may have resulted from the disorganized structure of the neuroepithelial loops in these regions. It is reasonable to assume that a disruption in the 3-dimensional signaling framework, as a result of the culture system, may effect the normal development of the transdifferentiated retina. This may therefore lead to the displacement of certain cell types in some regions of the transdifferentiated neuroepithelium.

The fact that bFGF-treated RPE explants expressed both HuD and Islet-1, but non-treated explants did not, would suggest that either of these markers are clear and accurate markers of transdifferentiation. These markers may be of particular use given that they are expressed in all fully-transdifferentiated RPE explants, which may be a consequence of the fact that ganglion cells and amacrine cells are known to differentiate before other retinal specific cell types (Cepko et al., 1996, Prada et al., 1992, Prada et al., 1991). For this reason, it is perhaps reasonable to assume that these markers will be expressed at an early stage in the development of the transdifferentiated neuroepithelium, and therefore more likely to be observed, if, in some cases, the development of the neuroepithelium is for some reason impaired.

HuD is a particularly good marker of transdifferentiation as it is robustly expressed in the resulting neuroepithelium. It is also cytosolic, unlike nuclear markers, Pax6 and Sox2, which means it can be easily identified, where background signal in the nucleus can often be incorrectly mistaken for positive expression of nuclear markers, given that this is the only part of the cell not obscured by heavy pigment in RPE. Additionally, HuD is preferentially expressed on the inverted inner edge of the neuroepithlium of transdifferentiated retina, in contrast to native retina, which provides supplementary information as to the origin of the neuroepithelium in these cultures. Native retina by definition will have a normal orientation and therefore, this marker is useful in identifying contaminant retina should any accidentally remain, post-dissection.

3.3.4.3 Otx2 & CRX:

In contrast to HuD and Islet-1 expression, CRX and Otx2 expression in transdifferentiated retina was largely localized to a layer adjacent to the outer edge of the neuroepithelium, consistent with where one would expect the development of the outer nuclear layer in this apparently inverted presumptive retina. Indeed, CRX is widely-regarded as one of the key transcription factors responsible for the differentiation of photoreceptors.

Mutations in the gene are known to be associated with severe photoreceptor diseases, including Leber's Congenital Amaurosis (Swaroop et al., 1999, Freund et al., 1997). In addition, CRX-/- mice have been observed to have problems in the extension of their rod outer-segments (Furukawa et al., 1999, Morrow et al., 2005, Furukawa et al., 1997). Over-expression of CRX in iris-derived cells has been shown to correlate with an increase in expression of a number of photoreceptor-specific proteins such as rhodopsin, recoverin, arrestin, and inter-photoreceptor-binding protein (IRBP) (Haruta et al., 2001, Akagi et al., 2005). Similarly, over-expression of CRX in mouse retina leads to an increase in rod photoreceptors which correlates with a decrease in the number of amacrine and Müller glia cells (Furukawa et al., 1999).

CRX has been reported in human cells to be initially expressed in proliferative cells and differentiating bipolar cells and then later becoming expressed at a higher level in photoreceptors in the mature human retina (Glubrecht et al., 2009) which is expected given the transcriptional control the transcription factor appears to exert over photoreceptor-specific genes. The reported expression of CRX in immature bipolar cells was based upon its co-localization with the bipolar cell marker CHX10, however, given that this marker is also known to label multi-potent neuroretinal progenitors in the developing neuroblastic layer, questions as to the specificity of this marker as a bipolar cell indicator could perhaps be raised. However, in accordance with CRX being reportedly expressed in bipolar cells, some less-strongly labeled cells were present in a more scattered fashion towards the middle of the transdifferentiated neuroepithelium where bipolar cells are likely to reside. Further analysis

would be required in order to confirm this. The fact that most of the expression of CRX in transdifferentiated RPE cells appears towards the outer edge of the neuroepithelium would suggest that following 7 days treatment with bFGF, the transdifferentiated retina is at a developmental stage which is more akin to that of the adult retina, where CRX expression is strongest in the developing photoreceptor cells of the ONL (Glubrecht et al., 2009).

As expected of a neuroepithelium at a similar stage, Otx2 displayed a similar pattern of expression and would most-likely co-localise with the expression of CRX. During development, Otx2 is initially expressed in the outer layer of the optic cup in the presumptive RPE cells and is heavily implicated in the proper development and maintenance of the RPE given that its expression is maintained in the RPE until adulthood (Bovolenta et al., 1997, Baas et al., 2000, Fujimura et al., 2009, Martinez-Morales et al., 2003, Martinez-Morales et al., 2004, Martinez-Morales et al., 2001, Rath et al., 2007, Muller et al., 2007, Larsen et al., 2009, Glubrecht et al., 2009, Beby et al., 2010). Loss of function of the protein has been associated with a number of different eye malformations including micropthalmia and anopthalmia (Verma and Fitzpatrick, 2007, Acampora et al., 1995, Ang et al., 1996, Matsuo et al., 1995, Martinez-Morales et al., 2001, Beby et al., 2010). In addition to this, several studies have reported evidence that Otx2 is important for transactivation of a number of different RPE-specific genes including DOPAchrome tautomerase (DCT) involved in melanin-synthesis (Takeda et al., 2003), Bestrophin (Esumi et al., 2009), and Mitf (Westenskow et al., Martinez-Morales et al., 2004, Martinez-Morales et al., 2003).

The role of Otx2 appears to play in specification of the RPE is reflected in the restricted expression patterns of the transcription factor at early developmental stages including the dorsal optic vesicle and the outer optic cup which are destined to becoming RPE (Bovolenta et al., 1997). However, at later stages, Otx2 begins to be expressed in the developing neuroepithelium. The spatio-temporal expression of Otx2 is complicated and it has been implicated in the specification of most, if not all of the different retinal cell types (Bovolenta et al., 1997).

Otx2 was found to be expressed in a similar region to CRX, which was expressed in presumptive photoreceptor cells in a similar pattern to that observed in transdifferentiated embryonic chicken RPE (Glubrecht et al., 2009, Rath et al., 2007), including a few possible bipolar cells in the middle of the neuroepithelium. This would suggest that at some point in development, Otx2 and CRX are required for photoreceptor cell and bipolar cell specification, as has been suggested (Glubrecht et al., 2009, Bovolenta et al., 1997). Otx2 is known to be critical for photoreceptor differentiation in mice and has been reported to transactivate CRX expression (Nishida et al., 2003, Koike et al., 2007, Kimura et al., 2000). A loss in Otx2 expression in developing photoreceptors has been associated with a loss in photoreceptor cell fate and a consequent gain in amacrine cell fate (Nishida et al., 2003). Therefore, given the likely-hood that these transcription factors are co-expressed in transdifferentiated RPE, we can reasonably assume that at 7 days, the transdifferentiated neuroepithlium is at a developmental stage where photoreceptor progenitors have most-likely started to be born.

Embryonic chicken RPE explants not treated with bFGF were also observed to express both Otx2 and CRX transcription factors. In the case of Otx2, this is to be expected given the aforementioned role the protein is thought to play in the specification and maintenance of the RPE phenotype. However, less expected was the expression of CRX in pigmented RPE cells. It is possible that there is some cross-over in the binding of the antibody to Otx2, given the fact that there is a very high sequence similarity between the two transcription factors, and perhaps therefore this would also be reflected in the existence of similar structural epitopes. Despite this, several studies including our own, have reported the weak expression of CRX in RPE cells, including children of up to 4 years (Glubrecht et al., 2009, Esumi et al., 2009)(unpublished data). The reason for this observed expression remains unclear, however, it could perhaps reflect the neuronal potential that RPE appear to possess.

3.3.4.4 Rhodopsin:

The expression patterns of CRX and Otx2 in presumptive photoreceptors, largely corresponds with the expression of rhodopsin in the membranes of a number of cells in the outer edge of the novel retina. This again confirms the inverted nature of the neuroepithelium, and suggests that transdifferentiated retina can reach later stages of development, where crucial molecular components of functional machinery are present.

Untreated RPE explants were negative for rhodopsin expression consistent with maintenance of the RPE phenotype. It seems likely that the exogenous factors added to the RPE explants can account for the lack of surrounding tissues usually involved in the development of the retina *in* vivo. This is encouraging as many studies have focused on the early developmental markers expressed in the transdifferentiated retina, rather than functional proteins.

This is, to our knowledge, the first time anyone has observed the expression of rhodopsin in the chicken model of RPE transdifferentiation, with the only other report coming from transdifferentiated rat RPE cells (Zhao et al., 1995). The expression of mature markers of differentiation would suggest that these cells are no longer in a progenitor state, have exited the cell cycle of begun to differentiate (Jadhav et al., 2006, Nelson et al., 2007, Marquardt et al., 2001). Only a small number of cells were observed to be expressing rhodopsin following 7 days in culture, which may suggest that the photoreceptor progenitors within the transdifferentiated explants were only just beginning to differentiate into mature photoreceptors. This is also reflected in the weak band detected in positive RT-PCR data. The absence of rhodopsin positive cells in some explants could therefore result from different regions of the neuroepithelial loops developing at slightly different rates. In addition, the lack of rhodopsin expression detected in some RT-PCR preparations may be accounted for by the fact that these explants appeared to be in a less healthy state following 7 days in culture (data not shown), as previously discussed (see experiment 3.2). Interestingly, rhodopsin expression *in ovo* is not normally found in the retina until very late in its development [reportedly E14

onwards in the chicken (Jacob et al., 2005)]. Therefore, if transdifferentiated retina develops at a similar rate to the native retina, RPE cells taken from E4 embryos and treated with bFGF for 7 days in culture would only be expected to have reached the equivalent of E11 retina *in ovo*. Rhodopsin expression is reportedly absent in the retina at this stage, which may suggest that the transdifferentiated retina, in this system, or at least regions of the neuroepithelium, is able to develop at a more rapid rate than its native counterpart, and initiate early onset of rod differentiation.

It could be that something about this culture system biases the differentiation of retinal progenitors towards a photoreceptor fate so that they are observed to develop more quickly. The fact that the transdifferentiated retina develops in a different shape to that of the native retina may mean that there are different micro-environmental, developmental cues in different regions of the explants. This could potentially account for the clustering of rhodopsin positive cells observed in some areas, with none in others. Other studies of chicken RPE transdifferentiation have reported the presence of cone markers such as visinin, a calcium binding protein similar to mammalian recoverin, which is found in cone photoreceptors (Li et al., 2010, Liang et al., 2006a, Yan and Wang, 1998, Sakami et al., 2008). Rod progenitors, if not more mature rods, can therefore be produced via transdifferentiation of RPE cells. This is encouraging evidence that RPE transdifferentiation could be used to produce new photoreceptors for transplantation into diseases involving retinal degeneration, providing the process can be replicated in humans (MacLaren et al., 2006, Pearson et al., 2012, Gust and Reh, 2011).

3.3.4.5 General discussion:

Transdifferentiated RPE as a result of prolonged treatment with bFGF is able to produce new retina that expresses a number of different markers of developing retina. For the most part, the development of this neuroepithelium appears to closely resemble that of native retina in terms of the spatial—temporal expression of these markers, albeit with a perhaps

slightly early expression of some mature markers, potentially as a result of a lack of surrounding tissues. Transdifferentiated retina could therefore be a useful tool in the study of retinal development, as well as a source of apparently well-developed retinal cells for transplantation.

3.4 Confirmation of the stage at which bFGF-mediated transdifferentiation becomes restricted:

3.4.1 Introduction:

It is important to ascertain the developmental stage at which transdifferentiation can, and cannot take place in response to bFGF treatment. This is so changes that may take place during the development of the RPE, which restrict the capacity for transdifferentiation, might be identified. In addition, it will be necessary to establish a model system in order to investigate the potential of various factors to extend the window of competence for transdifferentiation, so that later stage RPE, such as the available human RPE tissue, might be coaxed to undergo the phenomenon. It has been reported that the ability to transdifferentiate in response to bFGF is lost in the RPE at approximately E5, stage HH25 (Sakami et al., 2008), however, given the fact that these studies were not optimized for human cells, it will be important to investigate whether or not the unspecified components of HESC medium are able to hinder, or promote the transdifferentiation of the RPE at different developmental stages. As a control, it will be necessary to employ the use of a medium that was previously used in the study of the bFGF-sensitive window, in which transdifferentiation can take place. RPE was reported to lose this capacity at stage HH25 in this medium (Pittack et al., 1997, Pittack et al., 1991).

This study will therefore hope to establish an experimental model for investigation into the capacity for RPE transdifferentiation, as well as to investigate whether RPE cultured in HESC medium is able to behave in a similar manner to that of standard chicken media.

3.4.2 Materials & Methods:

Embryonic chick RPE of several developmental stages was tested for the capacity to transdifferentiate in response to bFGF in the standard, transdifferentiation culture system described in chapter 2. In addition to HH24, a stage at which transdifferentiation in response to 100ng/ml treatment with bFGF is robust, several other stages, including HH25 and HH27, which are reportedly past the point in development at which embryonic chicken RPE can transdifferentiate in response to bFGF.

Each RPE sheet was divided into 4 separate explants with two being cultured in standard HESC medium, and the other two being cultured in a control medium (DMEM/F12 + 1% FBS). One explant in each culture medium was subsequently treated with bFGF (100ng/ml) to attempt to induce transdifferentiation, whereas the remaining two were untreated as negative controls. By splitting single RPE monolayers from a single embryo into four sections, it was possible to have developmental stage-matched RPE for each culture condition.

Immunohistochemical analysis was performed as described in chapter 2.4.

3.4.3 Results:

In addition to observing classical transdifferentiation in chick RPE explants HH24, chick RPE explants from later developmental stages, including HH25 and HH27, also displayed evidence for undergoing the phenomenon in response to bFGF, in contrast to previous reports in the literature (Fig. 3.21i/ii for example). This was found to be the case for

chick RPE explants cultured in both HESC medium (Fig. 3.21i/iiA-N) as well as the control medium, DMEM/F12+1%FBS, used in previous reports of chick RPE transdifferentiation (Fig. 3.21iiO-Ci). Despite not being observed to express retinal progenitor markers, Sox2 (Fig. 3.21iB, F) or Pax6 (Fig. 3.21iC, G) in either bFGF treated, or untreated HH27 chick RPE explants cultured in HESC medium, somewhat pigmented, multi-layered, thickened, neuroepithelium-like structures, with pigment granules throughout, were visible in response to bFGF (Fig. 3.21iA, D). However, this was not found to be the case in the single-layered, thinner, untreated RPE explants (Fig. 3.21iE, H). Additionally, another region of the bFGF treated, HH27, chick RPE explant displayed limited HuD expression in a non-pigmented, neuroepithelial structure, which was continuous with the pigmented RPE monolayer (Fig. 3.21iI-K). The pigment granules present in the neuroepithelial structure suggest a previous specification as RPE (Fig. 3.21iN). No HuD expression was observed in untreated controls, which retained a characteristically pigmented RPE phenotype (Fig. 3.21iL-M).

Similarly, areas of apparent transdifferentiation were also observed in chick RPE HH27, treated with bFGF in DMEM/F12+1% FBS control medium (Fig. 3.21iiO-Ci). A thickened region of multi-layered, largely non-pigmented, neuroepithelium was observed in a portion of the bFGF treated explant (Fig. 3.21ii O, R, W, Z). No such structure was visible in untreated explants, which retained a single layer of pigmented RPE cells following 7 days in non-adherent culture (Fig. 3.21iiS, V, Ai, Ci). In this instance, the neuroepithelial structure did display both Pax6 (Fig. 3.21iiQ) and Sox2 (Fig. 3.21iiP) expression in response to bFGF. Many of the cells in this region expressed both markers, indicating the presence of retinal progenitors, which also characteristically result from RPE transdifferentiation of HH24 RPE, as previously described. In addition to the presence of retinal progenitors, the expression of HuD, a marker of differentiating ganglion and amacrine cell development, in bFGF treated explants, implies that transdifferentiation has occurred in these explants to produce a number of differentiated retinal cell types (Fig. 3.21iiX). The majority of the HuD expression appeared to be localized to the basal side of the neuroepithelial region, in a manner similar to that observed for transdifferentiated HH24 RPE explants (Fig. 3.21iiX).

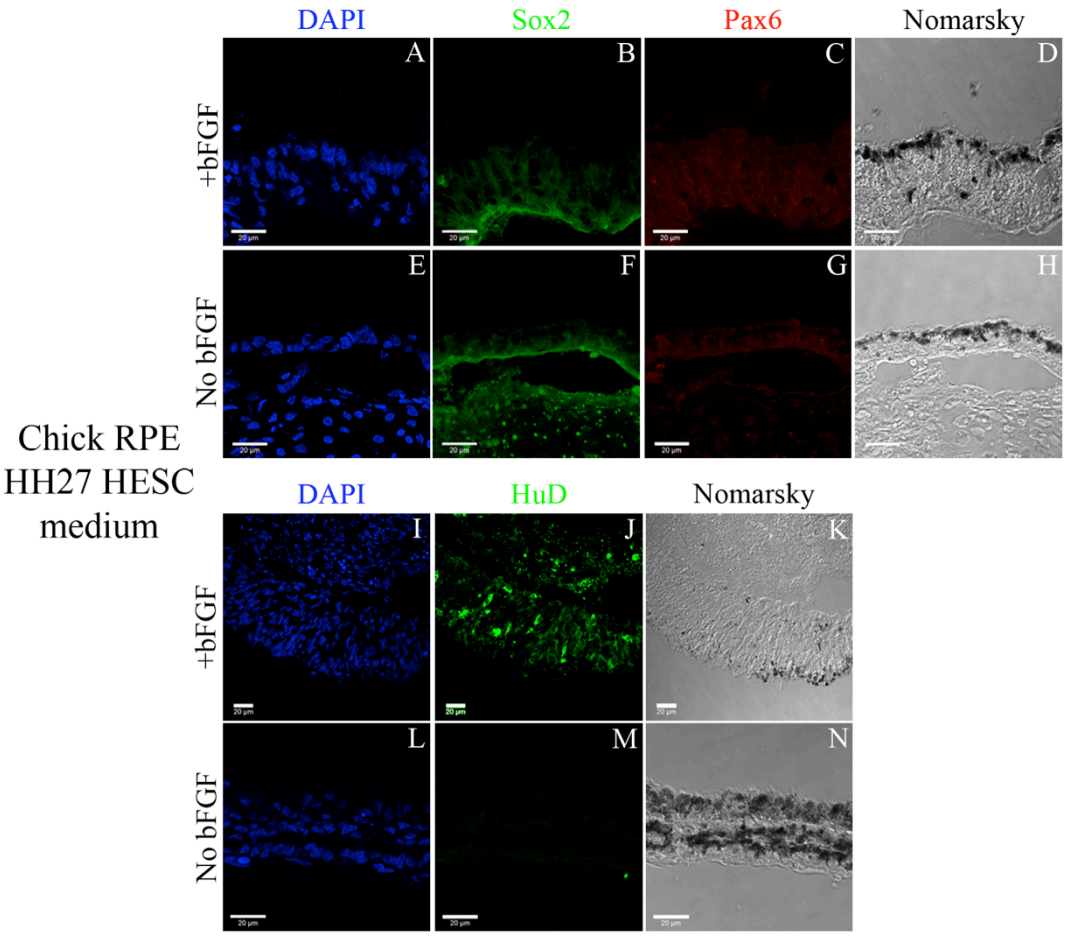


Fig. 3.21i
Chick RPE HH27 +/- bFGF 7 days. HESC medium - with extraocular mesenchyme.
There is evidence to suggest that some regions of RPE explants can undergo transdifferentiation in response to bFGF in both HESC and control media, even at later stages of development beyond HH24. For example, in HESC medium, a thickened region resembling neuroepithelium were observed in bFGF treated RPE (D) but were not positive for Pax6 (C) or Sox2 (D). However, another region of thickened, non-pigmented neuroepithelial-like tissue was HuD positive (J), and continuous with pigmented RPE. No retinal markers were expressed in negative controls (E-H, L-N). Scale bars: 20uM

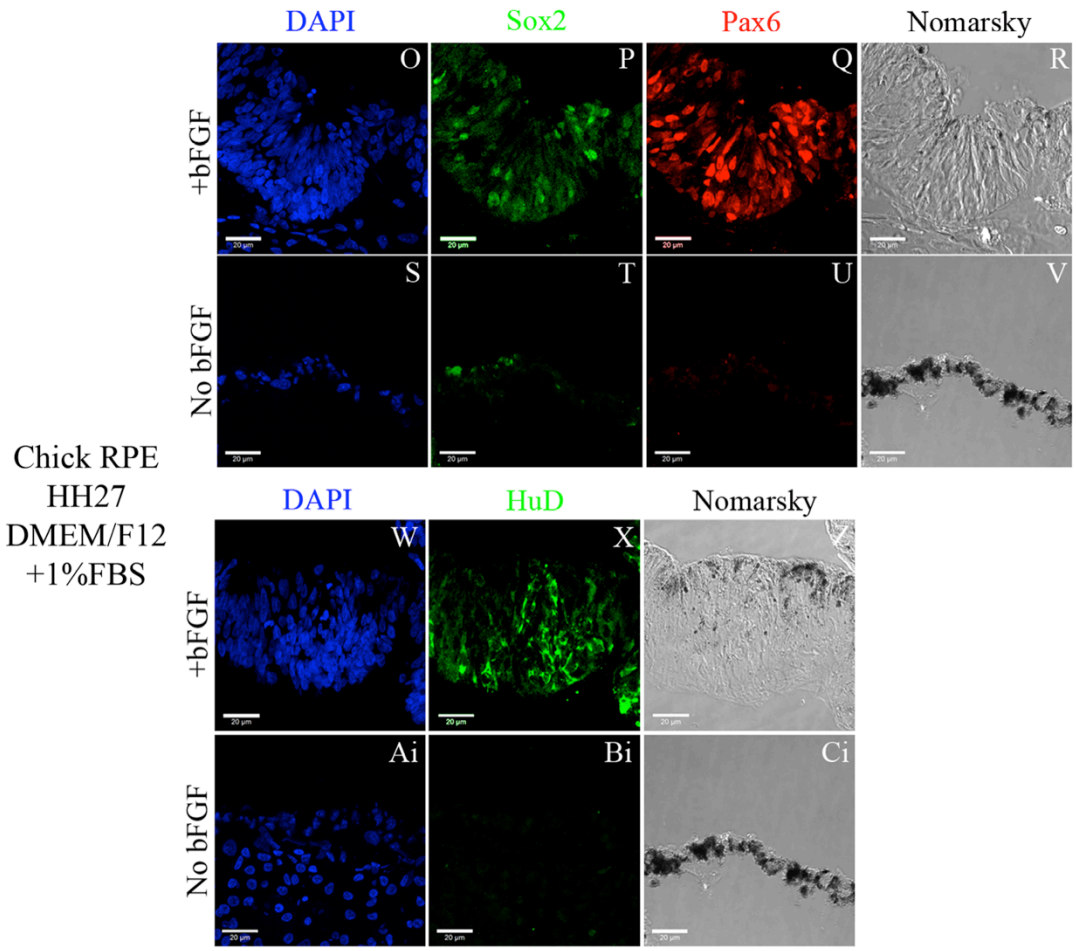


Fig. 3.21ii
Chick RPE HH27 +/- bFGF 7 days. DMEM/F12 + 1% FBS control medium - with extraocular mesenchyme.
Like that observed in HESC medium, a region of thickened, non-pigmented neuroepithelium was observed in bFGF treated RPE cultured in DMEM/F12+1% FBS medium (R, Z). This region was both Pax6 (Q), HuD (X), and Sox2 (P) positive, implying that transdifferentiation has taken place. No retinal markers were expressed in negative controls without bFGF, and maintained a pigmented monolayer phenotype. Fluoresence digitally enhanced. Scale bars: 20um.

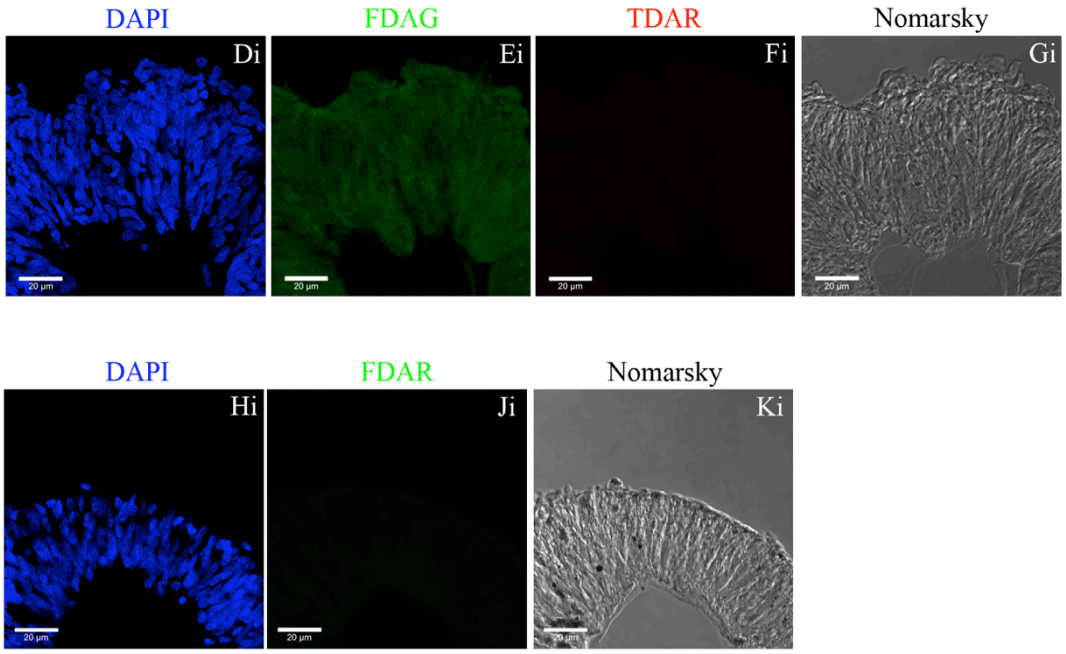


Fig. 3.22
Chick RPE HH27 +bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS no primary antibody negative controls.
No signal was detected in no primary antibody negative controls when transdifferentiated retina was labelled, except for some background autofluorescent signal detected in the FITC channel (Ei). Scale bars: 20um.

Once again, a number of HuD positive cells were also located within the middle of the neuroepithelium, once again like that of HH24 transdifferentiated RPE (Fig. 3.21iiX). No HuD expression was observed in HH27 RPE explants cultured in DMEM/F12+1% FBS without the exogenous addition of bFGF after 7 days (Fig. 3.21iiBi). No signal was detected above normal background in tissue labeled with secondary antibodies only, which implies that the signal in samples first incubated with primary antibodies are as a result of primary antibody binding (Fig. 3.22).

3.4.4 Discussion:

Surprisingly, some regions of RPE as late as HH27 were observed to display characteristics of transdifferentiation when treated with bFGF in both media. Several areas of these explants, as well as others at less mature developmental stages after HH25, were observed to exhibit regions which appeared to have a neuroepithelial, or pseudo-neuroepithelial, structures within bFGF treated explants. In some cases, these regions, which appeared to contain a low number of pigment granules, which would imply a previous specification as an RPE monolayer. Additionally, these regions were often observed to express retina markers such as Pax6, Sox2 and HuD in a variable fashion, which did not appear to be dependent on which basal medium was employed. This apparent transdifferentiation was contrary to previous reports that chicken RPE cannot undergo bFGF-induced transdifferentiation past stage HH24 (Park and Hollenberg, 1993, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Pittack et al., 1991, Pittack et al., 1997, Sakami et al., 2008). Given the fact that the window in which the capacity for transdifferentiation was relatively well established in the literature, it was thought that these apparently retinal structures in bFGF treated cultures may result from contaminant retinal or mesenchymal tissue, or indeed as a feature of the damage induced by the initial dissection. This was despite

the fact that the observed neuroepithelial structures were continuous with pigmented RPE monolayers in some places, which implies transdifferentiation has taken place.

In order to confirm that these retinal structures resulted from the transdifferentiation of RPE cells, it was necessary to develop techniques to obtain pure sheets of RPE before culture, in addition to analysis of the expression profile of RPE sheets post-dissection.

3.5 Confirmation of the stage at which RPE transdifferentiation in response to bFGF becomes developmentally restricted – dispase treatment of explants.

3.5.1 Introduction:

In order to confirm that the expression of retinal markers in response to bFGF did not occur as a result of the presence of contaminant material, it was necessary to repeat the study following light, enzymatic removal of attached tissues, primarily the extra-ocular mesenchyme at the basal surface of the RPE monolayer. Additionally, it was necessary to characterize the expression pattern of RPE sheets immediately following dissection, in order to ensure that retinal marker expression was not initiated by the process of dissection. Rhodopsin expression was also analysed in these cultures in order to give additional indication of the level of transdifferentiation in response to bFGF.

3.5.2 Materials & Methods:

Embryonic chicken RPE at different developmental stages (HH24, HH25, HH26, HH27) was mechanically dissected as described in chapter 2, however, in this instance, eye globes were pre-treated with dispase solution to remove extra-ocular material as described in chapter 2. Removal of contaminant tissue was confirmed by immediate fixation and

immunhistochemical analysis of a randomly selected batch of explants. Culture of RPE explants was performed in the same manner described in experiment 3.4 above.

Immunohistochemical analysis was performed as described in chapter 2.4.

3.5.3 Results:

A series of RPE dissected from different developmental stages, HH21-HH26 were fixed and analysed immediately, post-dissection. These sheets consisted of only pigmented RPE cells, which were observed to be negative for Sox2 (Fig. 3.23i/iiB, F, J, N, R, V), HuD (Fig. 3.24i/iiC, G, K, O, S, W) and rhodopsin (Fig. 3.24i/iiB, F, J, N, R, V) expression. No other non-pigmented cells were present, which would suggest that RPE monolayers were pure. Interestingly, Pax6 expression was observed in RPE cells up until stage, contrary to labeling observed in sectioned whole eyes. However, as previously discussed, *en face* labeling for Pax6 has found that the expression does not fall away as sharply as previously thought, but appears to gradually decline after HH25, which it is not possible to observe in section. Chick RPE explants of different developmental stages appeared to exhibit variable capacity for transdifferentiation in response to bFGF, in both HESC medium and DMEM/F12+1% FBS medium. The capacity for transdifferentiation appeared to be reduced with a progression of developmental stage of the RPE explant as expected. HH24 RPE explants always exhibited characteristics of RPE to neural retina transdifferentiation, as previously discussed (Fig. 3.25i; 3.25ii). This included the formation of non-pigmented, multi-layered, neuroepithelium in response to bFGF in both media (Fig. 3.25iA, I; 3.25iiA, I), which was not observed in pigmented, negative controls (Fig. 3.25iE, M, 3.25iiE, M). Neuroepithelium was observed to contain a number of nuclei that were positively labeled for both Pax6 (Fig. 3.25iD, L), and Sox2 (Fig. 3.25iC, K). Neither of these transcription factors were observed in untreated, negative control explants (Fig. 3.25iG, H, O, P). Additionally, as well as expressing markers of retinal progenitors, retinal cell specific markers HuD (Fig. 3.25iiD, L), and rhodopsin (Fig. 3.25iiK) were both observed in transdifferentiated retina.

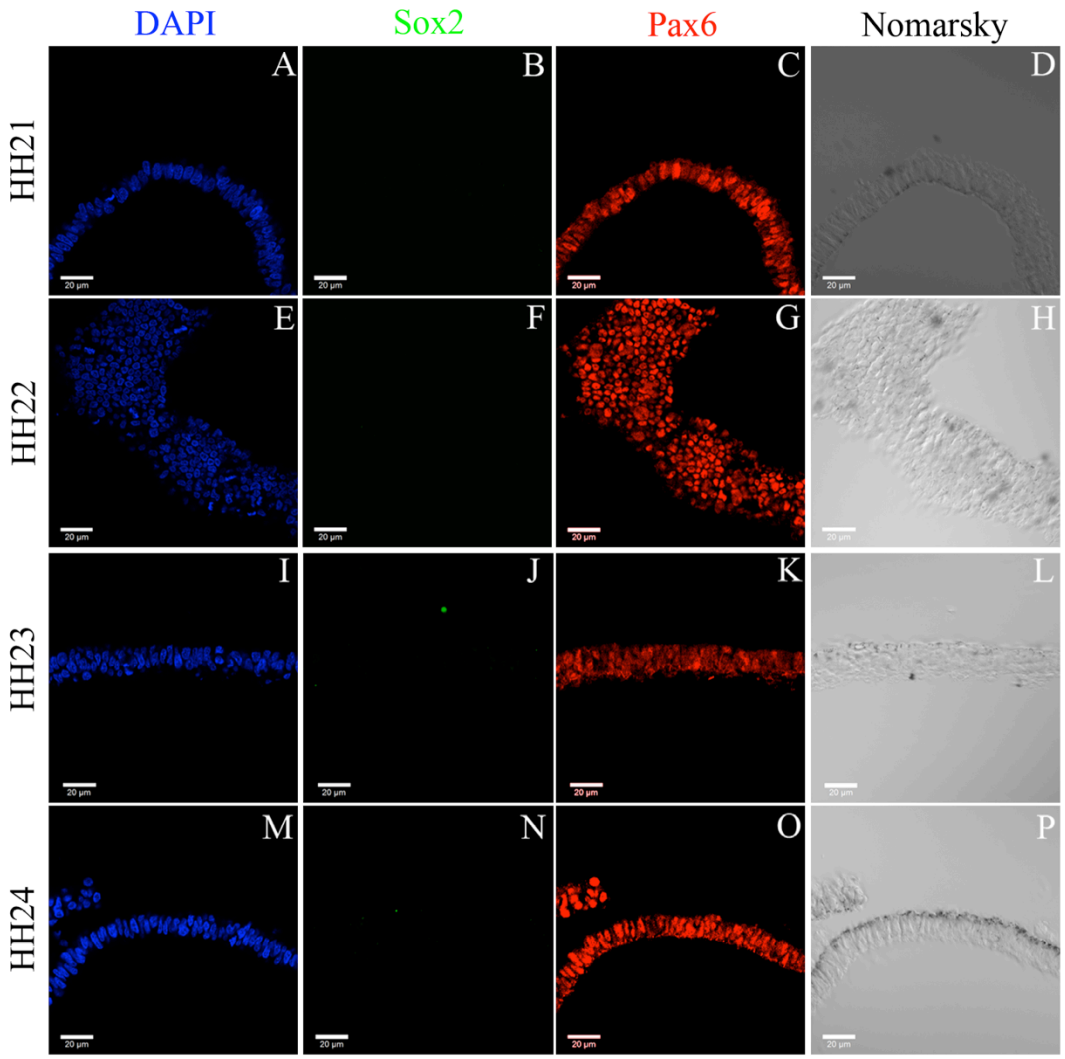


Fig. 3.23i

Chick RPE post-dissection controls following dispase treatment.

Dispase treated RPE explants were not found to express Sox2 (green) at any developmental stage (B, F, J, N, R, V). Pax6 (red) was observed in the RPE at earlier stages of development between HH21 and HH24 (C, G, K, O). Only pigmented RPE cells were present in explants, implying that all retinal cells and extraocular mesenchyme had been removed (D, H, L, P). Fluorescence digitally enhanced. Scale bars: 20um.

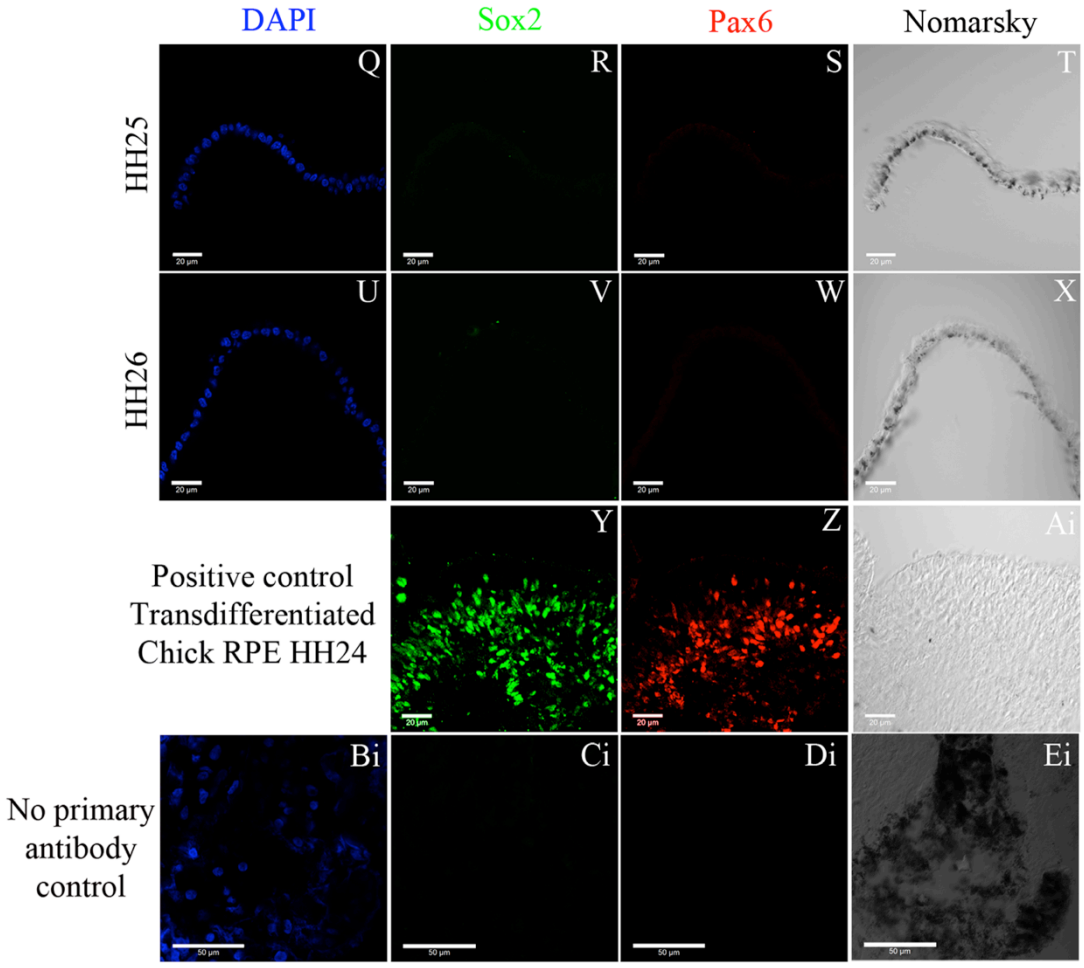


Fig. 3.23ii
Chick RPE post-dissection controls following dispase treatment.
Dispase treated RPE explants were not found to express Sox2 (green) at any developmental stage (R, V). Pax6 (red) was down-regulated in HH25 RPE onwards where Pax6 was absent frmo the RPE (S, W). Only pigmented RPE cells were present in explants, implying that all retinal cells and extraocular mesenchyme had been removed (T, X). Positive-labeling controls in transdifferentiated chick RPE HH24: Sox2 (Y), Pax6 (Z). No primary antibody negative controls (Bi-Ei). Fluoresence digitally enhanced. Scale bars: 20um.

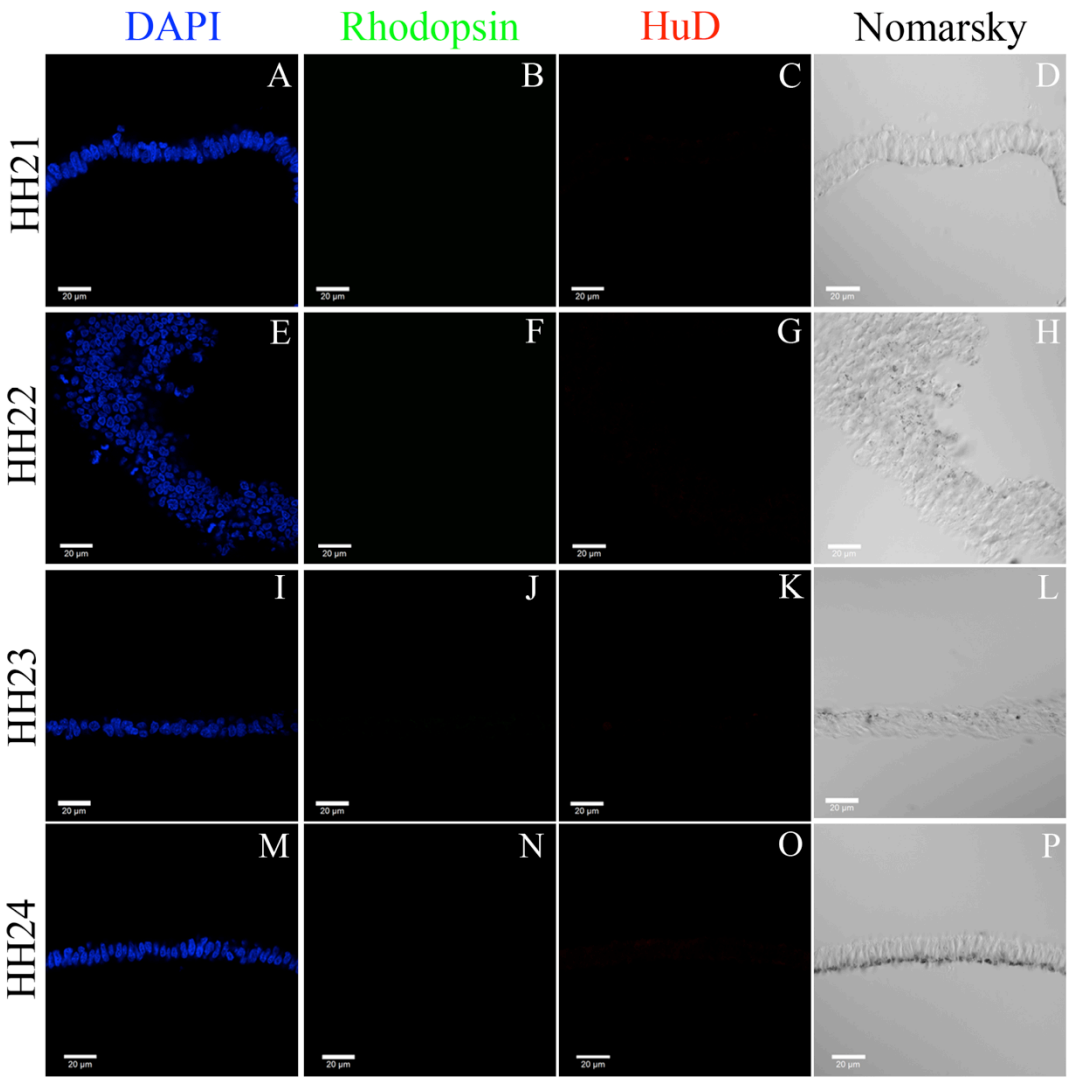


Fig. 3.24i
Chick RPE post-dissection controls HuD + Rhodopsin following dispase treatment.
Explants were fixed immediately following dissection. No RPE explants at any developmental stage HH21 - HH24 were observed to express either rhodopsin (Green) (B, F, J, N) or HuD (Red) (C, G, K, O). This is as expected for RPE cells which do not exhibit a neuroretinal phenotype charaterised by the expression of these markers. No non-pigmented cells were present at any stage HH21-HH24 in these control explants (D, H, L, P). Fluoresence digitally enhanced. Scale bars: 20um.

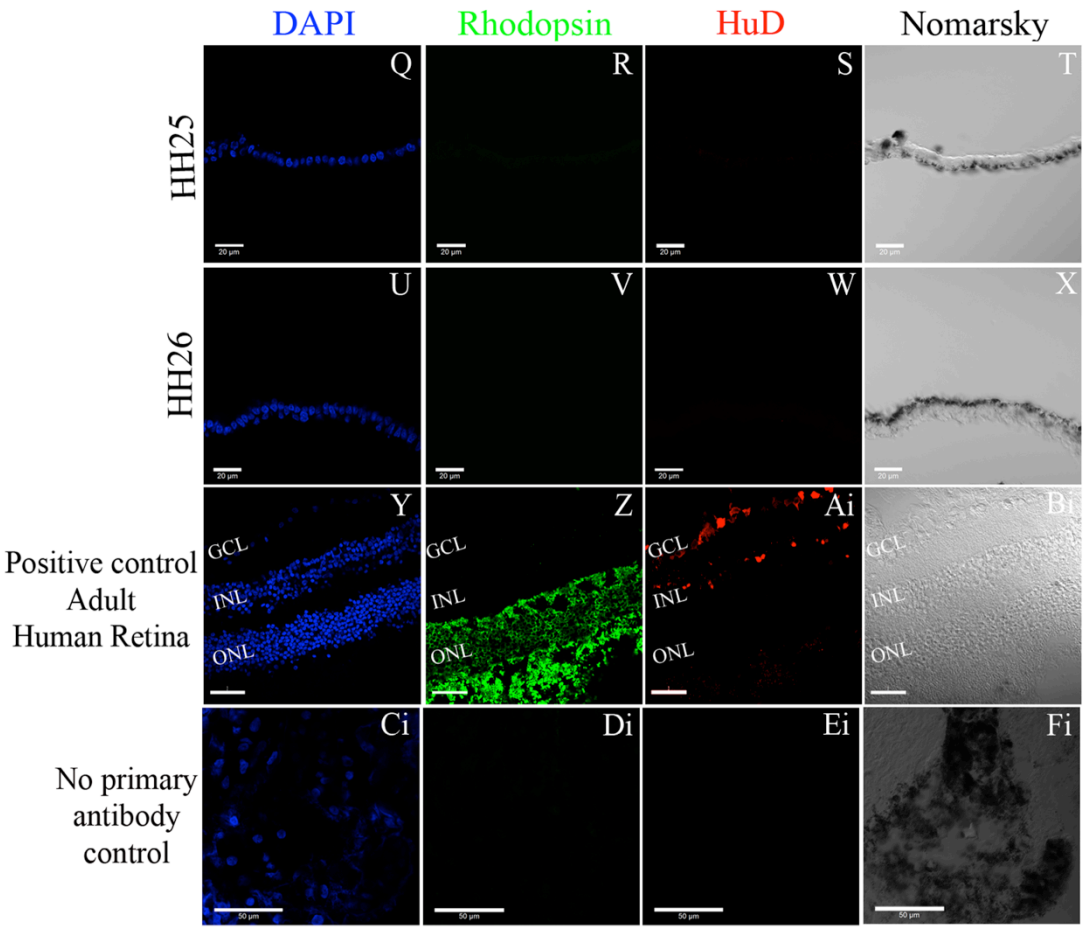


Fig. 3.24ii
Chick RPE post-dissection controls HuD + Rhodopsin following dispase treatment.
Explants were fixed immediately following dissection. No RPE explants at any developmental stage HH25-HH26 were observed to express either rhodopsin (green)(R, V) or HuD (red) (S, W). This is as expected for RPE cells which do not exhibit a neuroretinal phenotype charaterised by the expression of these markers. No non-pigmented cells were present at any stage HH25-HH26 in these control explants (T, X). Positive controls for fluorescent-labeling was adult human retinal tissue which, as expected, displayed positive labeling for rhodopsin (Z) in the outer nuclear layer (ONL), and HuD in the inner nuclear layer (INL) and Ganglion cell layer (GCL). No labeling was exhibited in no primary antibody controls for rhodopsin (Di) or HuD (Ai). Fluoresence digitally enhanced. Scale bars: 20um.

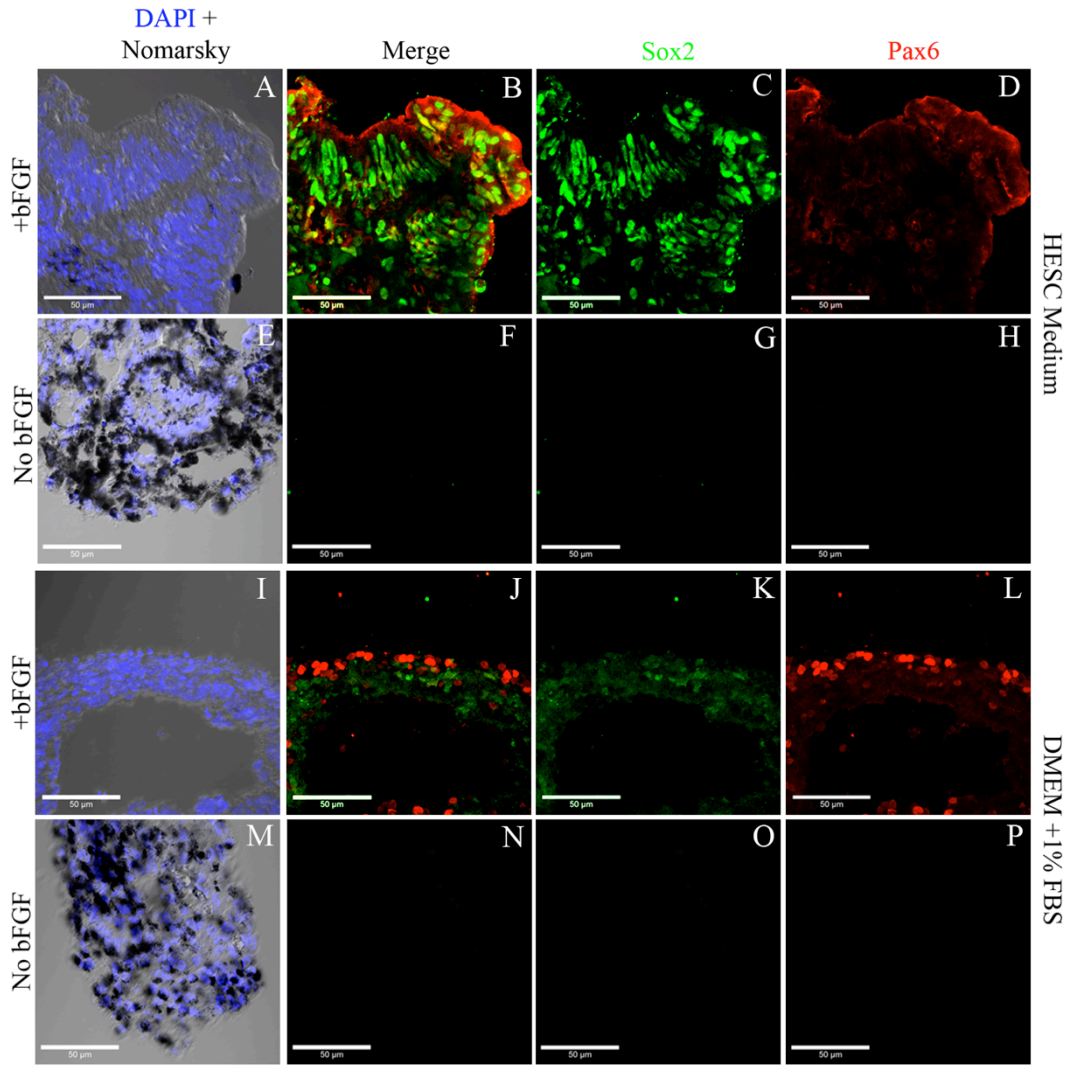


Fig. 3.25i
Chick RPE HH24 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Retinal progenitor cell markers.
Chick HH24 RPE explants exhibit evidence for transdifferentiation in both HESC medium and control (DMEM/F12+1% FBS) medium in response to bFGF. This included the formation of non-pigmented, neuroepithelium (A, I), which expressed retinal markers: Pax6 (D, L), Sox2 (C, K). No retinal markers were expressed in untreated negative controls (G, H, O, P), which remained pigmented (E, M). Fluoresence digitally enhanced. Scale bars: 50um.

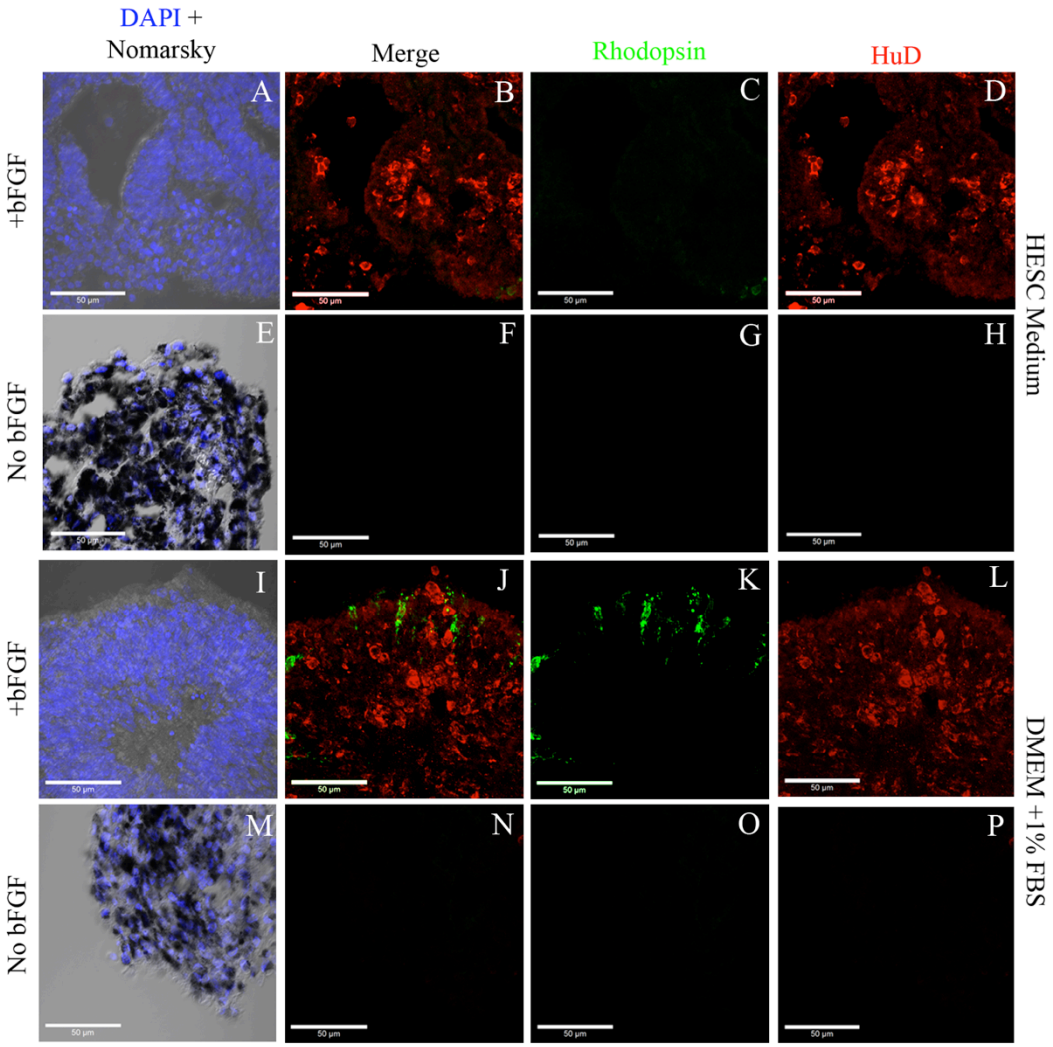


Fig. 3.25ii
Chick RPE stage HH24 +/- bFGF (100ng/ml) in DMEM/F12 + 1% FBS or HESC medium for 7 days - Retinal specific markers.
Transdifferentiation in response to bFGF treatment was observed at this developmental stage in both media after 7 days in culture. Transdifferentiation involved the formation of thickened neuroepithelial loops, which had undergone de-pigmentation (A, I), whereas untreated controls retained the pigmented RPE phenotype (E, M). Transdifferentiated RPE was observed to express HuD, indicating the presence of ganglion/amacrine cell differentiation (D, L), however, HuD expression was absent from untreated controls (H, P). Rhodopsin expression was observed in a small number of cells in some bFGF-treated explants, in both media (K). These were localised to the apical surface of the neuroepithelium, which demonstrates the inverted nature of the transdifferentiated neuroepithelium. Some transdifferentiated retinas did not express rhodopsin, but this was not specific to one medium (G). HESC medium (A-H), DMEM/F12 +1% FBS (I-P). Fluoresence digitally enhanced. Scale bars: 50uM.

HuD expression was localized to the cytoplasm of cells, which were largely found at the basal surface of the neuroepithelium, implying an inverted retinal phenotype, however, a number of cells throughout the neuroepithelium were observed to express the protein, possibly indicating the disorganized development of the novel retina (Fig. 3.25iiD, L). Similarly, the limited rhodopsin expression observed in a few cells of bFGF treated explants was always localized to the apical surface of the neuroepithelium, in cells of the presumptive photoreceptor layer (Fig. 3.25iiC, K). Rhodopsin was confined to the cell membrane of these cells, which is characteristic of the normal localization of rhodopsin, and a number of positive cells displayed a morphology reminiscent of developing photoreceptors (Fig. 3.25iiC, K). No rhodopsin or HuD expression was observed in untreated negative controls (Fig. 3.25iiG, H, O, P).

By HH25, chick RPE explants treated with bFGF appeared to display variability in the capacity for transdifferentiation, with some explants displaying evidence for the phenomenon, while others appeared to retain only limited capacity for transdifferentiation (Fig. 3.26i; 3.26ii, 3.27i, 3.27ii). For example, one explant displayed areas of somewhat de-pigmented regions which were quasi-neuroepithelial when cultured in the presence of bFGF in HESC medium (Fig. 3.26iA), when compared with the heavily pigmented, untreated negative control (Fig. 3.26iE). However, another region of explanted RPE sheet cultured in DMEM/F12+1% FBS displayed de-pigmentation, in addition to the formation of a region of neuroepithelial tissue which resembled a normal transdifferentiated retina more closely (Fig. 3.26iI). The untreated negative control in this medium largely retained a pigmented epithelial phenotype (Fig. 3.26iM). The neuroepithelium-like structure observed in HESC medium also expressed a limited amount of Sox2 in scattered nuclei (Fig. 3.26iC), as well as a large number of cells that expressed Pax6 in the non-pigmented region (Fig. 3.26iD). Some of these cells were found to express both Pax6 and Sox2 within the same nucleus, indicating the presence of retinal progenitors (Fig. 3.26iB). No Sox2 (Fig. 3.26iG) or Pax6 (Fig. 3.26iH) was observed in he untreated negative controls in HESC medium. The neuroepithelium observed in bFGF treated RPE in control medium (DMEM/F12 +1% FBS) was heavily

labeled for both Sox2 (Fig. 3.26iK) and Pax6 (Fig. 3.26iL) in the majority of cells of the neuroepithelium, in contrast with the structure observed in this HESC medium explant treated with bFGF (Fig. 3.26iC, D). Much of the Pax6 and Sox2 expression in control medium explants was observed to co-localise (Fig. 3.26iJ), once again indicating the presence of retinal progenitors in the neuroepithelium. Surprisingly, despite no Sox2 expression being observed in the negative control for explants cultured in DMEM/F12+1% FBS (Fig. 3.26iO), many of the cells in the pigmented explant did appear to exhibit the expression of Pax6 (Fig. 3.26iP). Both bFGF treated explants treated in HESC medium and control medium were both observed to express retinal amacrine/ganglion cell marker HuD (Fig. 3.26iiD, L). Fewer cells appeared to express the protein in this HESC medium explant (Fig. 3.26iiD), which displayed HuD expression in a few cells throughout this region, and retained more pigmentation and less of a neuroepithelial phenotype (Fig. 3.26iA; 3.26iiA), than the control medium explant, in which more cells expressed HuD, and were largely localized to the basal surface, indicating an inverted phenotype (Fig. 3.26iiL). This region in control medium also contained less pigmentation and an apparently better developed neuroepithelium more akin to the native retina (Fig. 3.26I; 3.26iiI). Only very limited signal for rhodopsin could be detected in either of the bFGF treated explants, in perhaps only one cell per explant (Fig. 3.26iiC, K). Interestingly, the localization of rhodopsin expression in transdifferentiated retina in control medium appeared to be located to the apical surface of the neuroepithelium (Fig. 3.26iiK), once again indicating the expected inverted phenotype. No HuD r rhodopsin expression was present in untreated control explants in either HESC medium (Fig. 3.26iiF-H)) or control medium (Fig. 3.26iiF-H).

To demonstrate the variability in the capacity for transdifferentiation at developmental stage HH25, another explant was observed to exhibit somewhat less of a capacity for transdifferentiation in response to bFGF (Fig. 3.27i; 3.27ii). Once again, the bFGF treated explant cultured in HESC medium displayed less pigmentation, in addition to a pseudo-neuroepithelium (Fig. 3.27iA, 3.27iiA), compared with untreated, negative control tissue, which retained a more pigmented phenotype (Fig. 3.27iE, 3.27iiE).

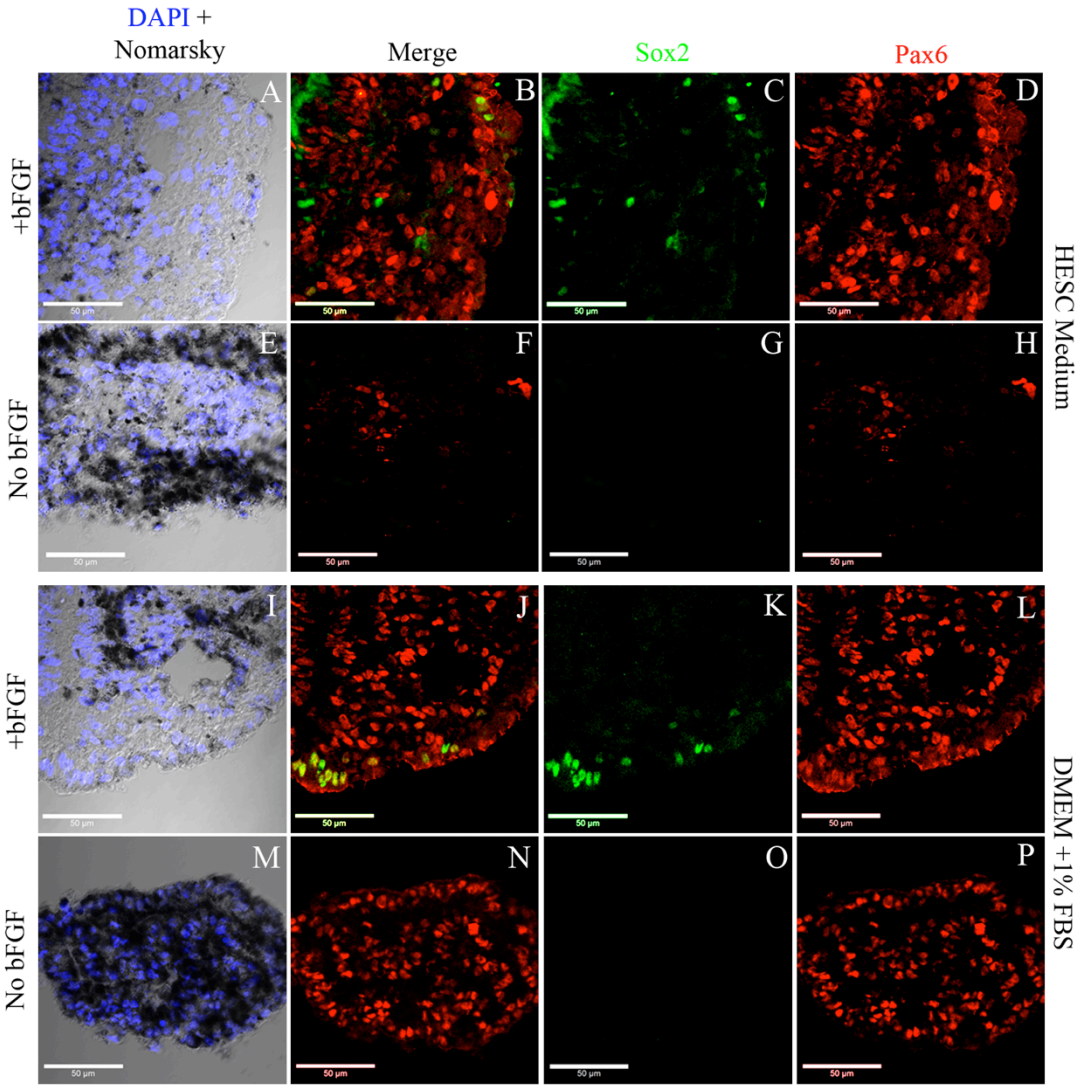


Fig. 3.27i
Chick RPE HH25 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 2, Less transdifferentiaiton - Retinal progenitor markers.
Capacity for transdifferentiation at HH25 appears to be variable. In this instance, the bFGF treated explant in HESC medium appeared to exhibit the best transdifferentiation, however, this wasn't particularly robust. Both bFGF explants in either medium exhibited pseudo-neuroepithelia (A, I) which had somewhat de-pigmented compared with negative controls (E, M). Robust Pax6 was observed throughout both explants (D, L) but Sox2 was only expressed in a few cells in either explant (C, K). Untreated, negative controls were negative for Sox2 as expected, however, some Pax6 expression was retained after 7 days in both media (H, P) These cells were robustly pigmented and appeared to retain the characteritic RPE phenotype (E, M). Fluroescense digitally enhanced. HESC medium (A-H), DMEM/F12 + 1% FBS controls medium (I-P). Scale bars: 50um.

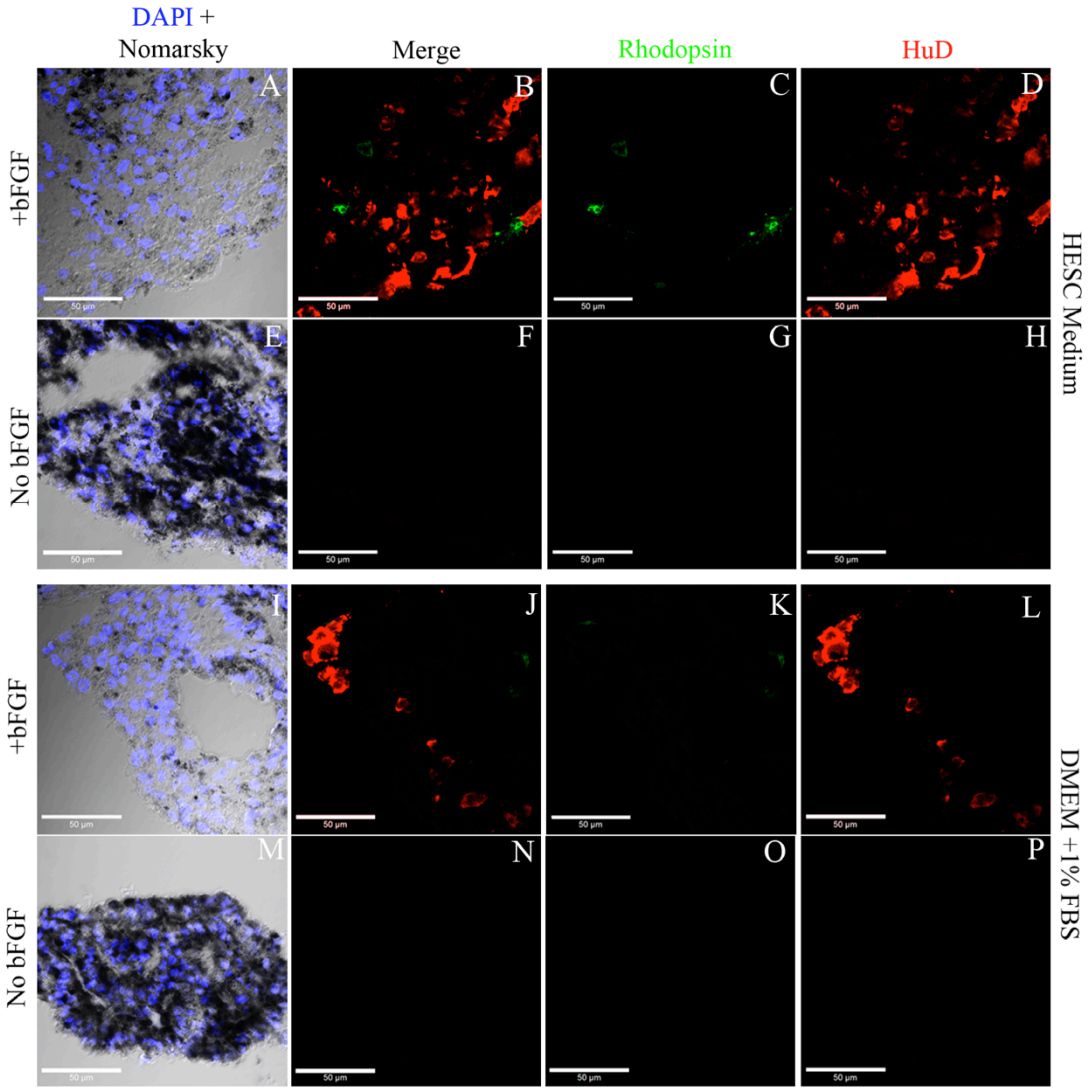


Fig. 3.27ii

Chick RPE HH25 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 2, Less transdifferentiaiton - Retinal cell specific markers.

These explants are an example of the variability in the capacity for transdifferentiation post-HH24 because only limited evidence for transdifferentiation was observed in response to bFGF. This included a level of de-pigmentation, and the formation of a pseudo-neuroepithelium in response to bFGF (A, I) in both culture media, when compared with untreated controls, which retained the characteristic heavy pigmentation associated with retention of the RPE phenotype (E, M). bFGF treated explants both exhibited some expression of HuD (D, L) suggesting that a level of retinal maturation had taken place in culture. Despite this, only very limited expression of rhodopsin was observed in any explnt at this stage, with most explants exhibiting no expression at all (C, K). Both HuD (H, P) and rhodopsin (G, O) were absent from all untreated explants. Fluorescence digitally enhanced. Scale bars: 50uM.

This region expressed Pax6 in the majority of nuclei (Fig. 3.27iD), which was often observed to co-localise with the few Sox2 nuclei, which were present (Fig. 3.27iB, C). No Sox2 expression was observed in the untreated negative control explant (Fig. 3.27iG), however, like untreated explants cultured in control medium in the previous experiment (Fig. 3.26iN, P), untreated controls exhibited some Pax6 positive nuclei throughout the explant (Fig. 3.27iH), with fewer Pax6 cells expressing the transcription factor in more pigmented regions (Fig. 3.27iE, H). bFGF treated explants in control medium displayed a similar phenotype with a de-pigmented pseudo-neuroepithelium having formed (Fig. 3.27iI), in contrast to the heavily pigmented RPE phenotype retained in untreated controls (Fig. 3.27iM). The bFGF treated explant was observed to express Pax6 throughout the explant (Fig. 3.27iL), however, Sox2 expression was limited to only a small cluster of nuclei in a few cells of the explant (Fig. 3.27iK), which all co-localised with Pax6 expression, indicating the presence of a limited number of retinal progenitors. No Sox2 expression was present in the untreated, negative, control, however, robust Pax6 expression was observed throughout the entire explant (Fig. 3.27iP), despite no evidence of transdifferentiation having been observed. Both bFGF treated explants cultured in HESC and control media were observed to express HuD (Fi. 3.27iiD, L respectively) within de-pigmented regions of the explant (Fig. 3.27iiA, I respectively), however, given the fact that the neuroepithelial structures appeared developmentally disorganized, this HuD expression did not appear to have a particular localization as it would in native retina. Similarly, a few rhodopsin positive cells were scattered throughout these regions with no particular organization apparent (Fig. 3.27iiC, K). This would suggest that a limited amount of transdifferentiation had taken place, particularly given that neither HuD or rhodopsin was observed in untreated, negative control explants in both HESC medium, and control medium (Fig. 3.27iiG, H, O, P).

Consistent with the idea that the capacity for transdifferentiation becomes more and more restricted with developmental stage, and that this potential is varied at each developmental stage, explants at HH26 showed variable evidence for the presence of transdifferentiation (Fig. 3.28i; 2.28ii, 3.29i; 3.29ii).

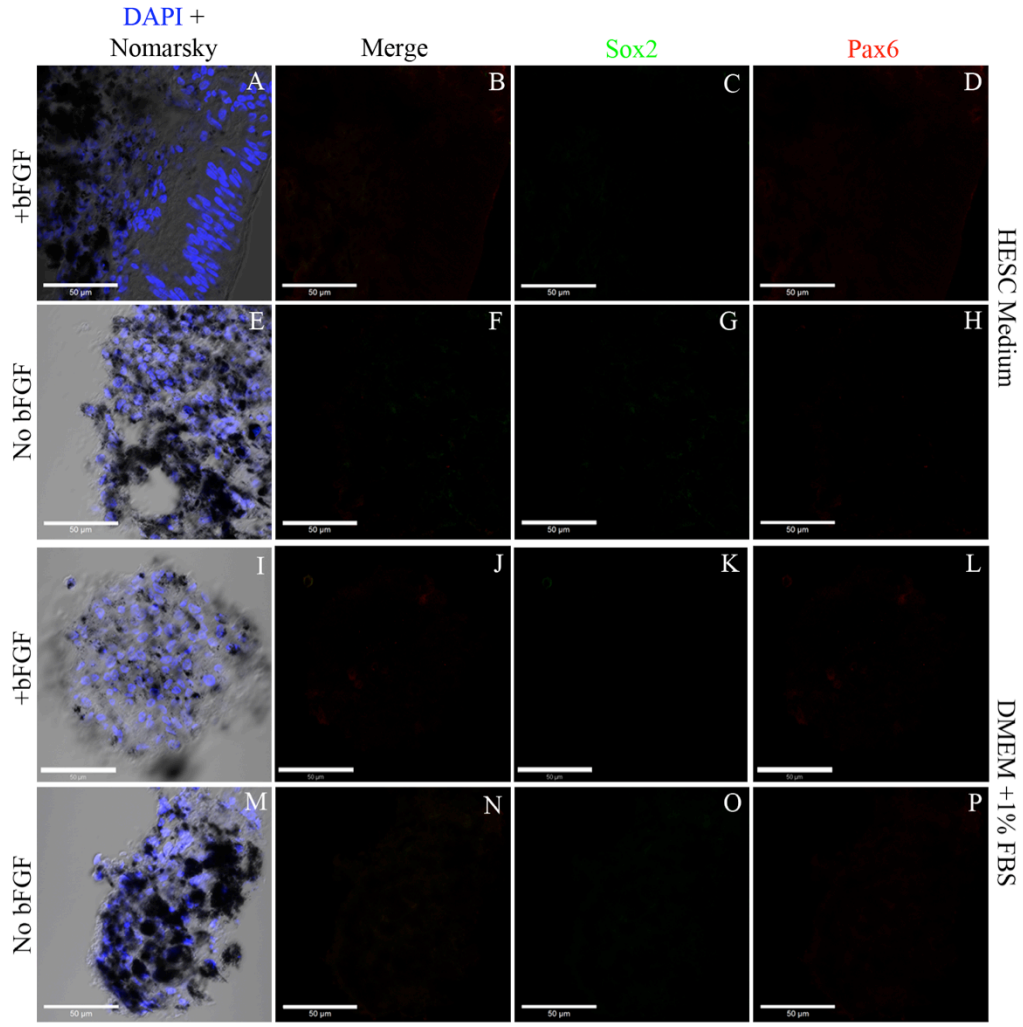


Fig. 3.28i

Chick RPE HH26 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 1, No transdifferentiation - Retinal progenitor markers.

The capacity for transdifferentiation continued to be variable at HH26, however, the capacity for transdifferentiation did appear to decrease at this later stage of development. This particular embryo did not exhibit transdifferentiation in response to bFGF in either HESC or control, DMEM/F12 + 1% FBS medium. Despite exhibiting a somewhat de-pigmented, pseudo-neuro-epithelium in HESC medium (A), no Pax6 or Sox2 expression was observed in this explant (C, D). No neuroepithelial structure as observed in bFGF treated RPE in control medium, which did not express retinal markers and largely retained its pigmentation (I-L). No retinal markers were present in untreated negative controls, which retained the pigmented RPE phenotype as expected (E-H, M-P). HESC medium (A-H), DMEM/F12 + 1% FBS medium (I-P). Scale bars: 50um.

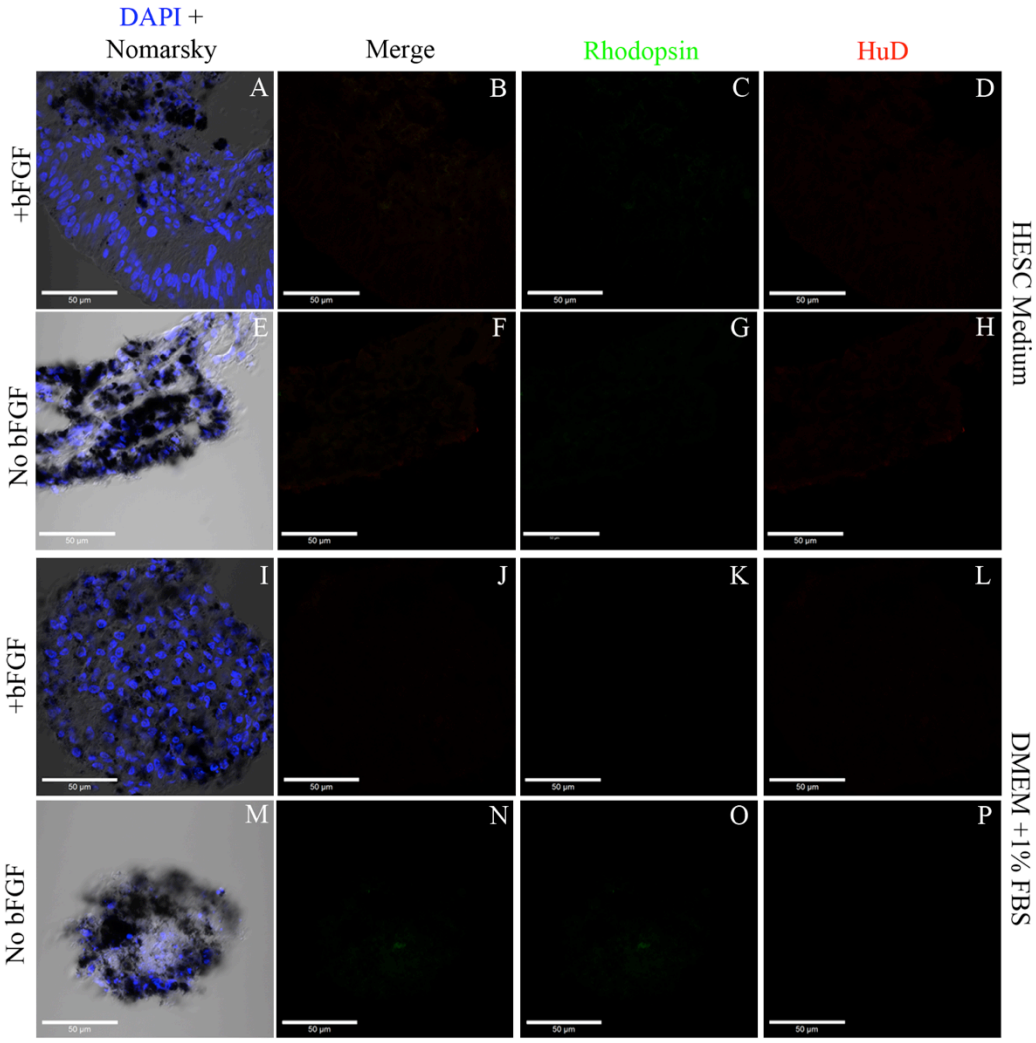


Fig. 3.28ii
Chick RPE HH26 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 1, No transdifferentiation - Retinal cell specific markers.
No retinal specific markers were observed in either bFGF treated, or untreated RPE explants from this particular embryo at this stage, despite a somewhat de-pigmented, neuroepithelial structure being observed in the bFGF-treated explant cultured in HESC medium (A). Both HuD (D, H, L, P) and rhodopsin (C, G, K, O) were absent from both bFGF treated and untreated explants, which suggests that no transdifferentiation has taken place in any explant. This is consistent with reports that chick RPE loses the capacity for transdifferentiation in response to bFGF treatment after stage HH24. HESC medium (A-H), DMEM/F12 + 1% FBS control medium. Scale bars: 50uM.

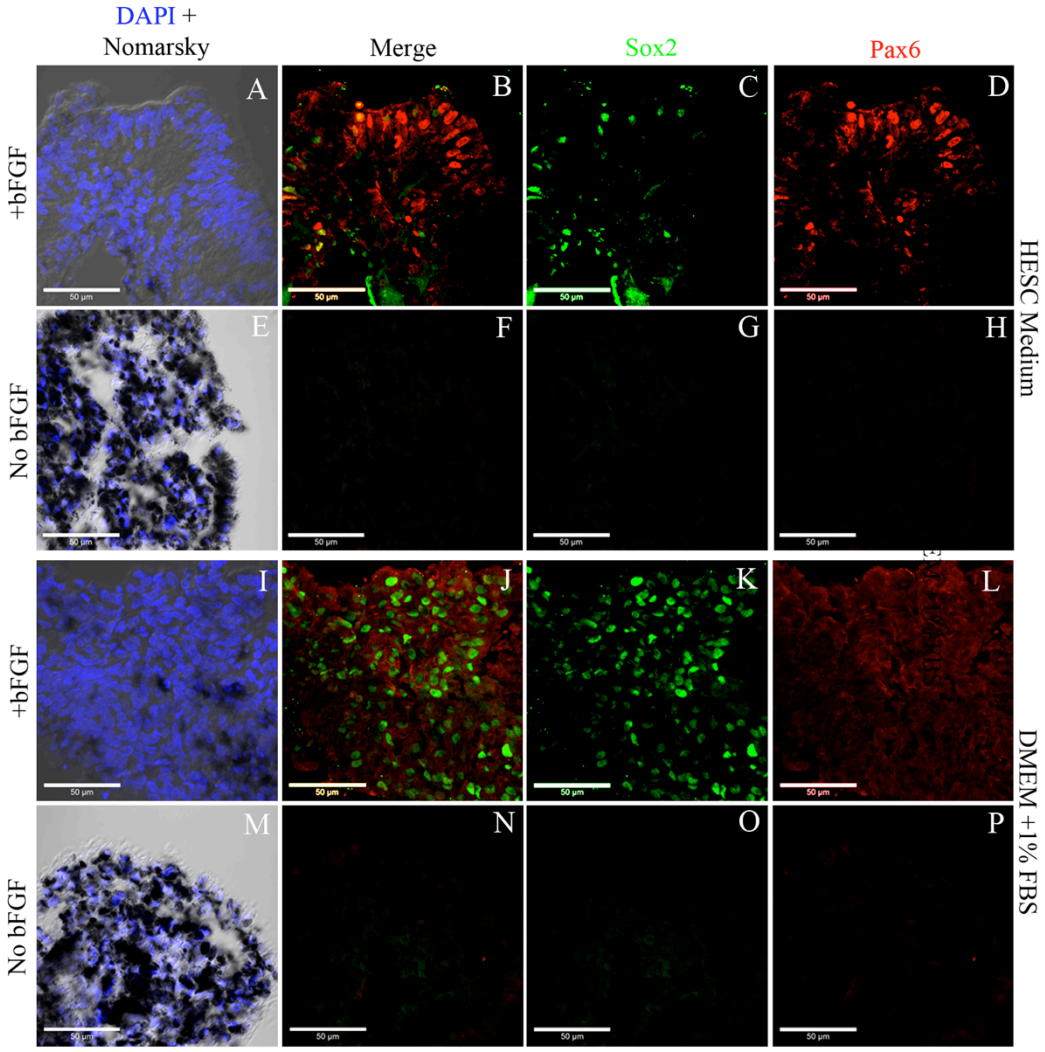


Fig. 3.29i

Chick RPE HH26 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 2, Some transdifferentiation - Retinal progenitor markers.

This embryo displayed some capacity for the RPE to undergo transdifferentiation in response to bFGF in both culture media. The RPE explant, +bFGF in HESC medium, formed a proper, non-pigmented, neuroepithelial structure (A), which expressed Pax6 (D) amd Sox2 (C). +bFGF RPE in control medium formed a pseudo-neuroepithelial structure (I), which robustly expressed Sox2 (K), with very limited Pax6 (L) expression. Untreated controls retained heavily pigmented RPE phenotypes, which did not express any retinal markers (F-H, N,-P). Fluorescence digitally enhanced. HESC medium (A-H), DMEM/F12 + 1% FBS control medium I-P). Scale bars: 50um.

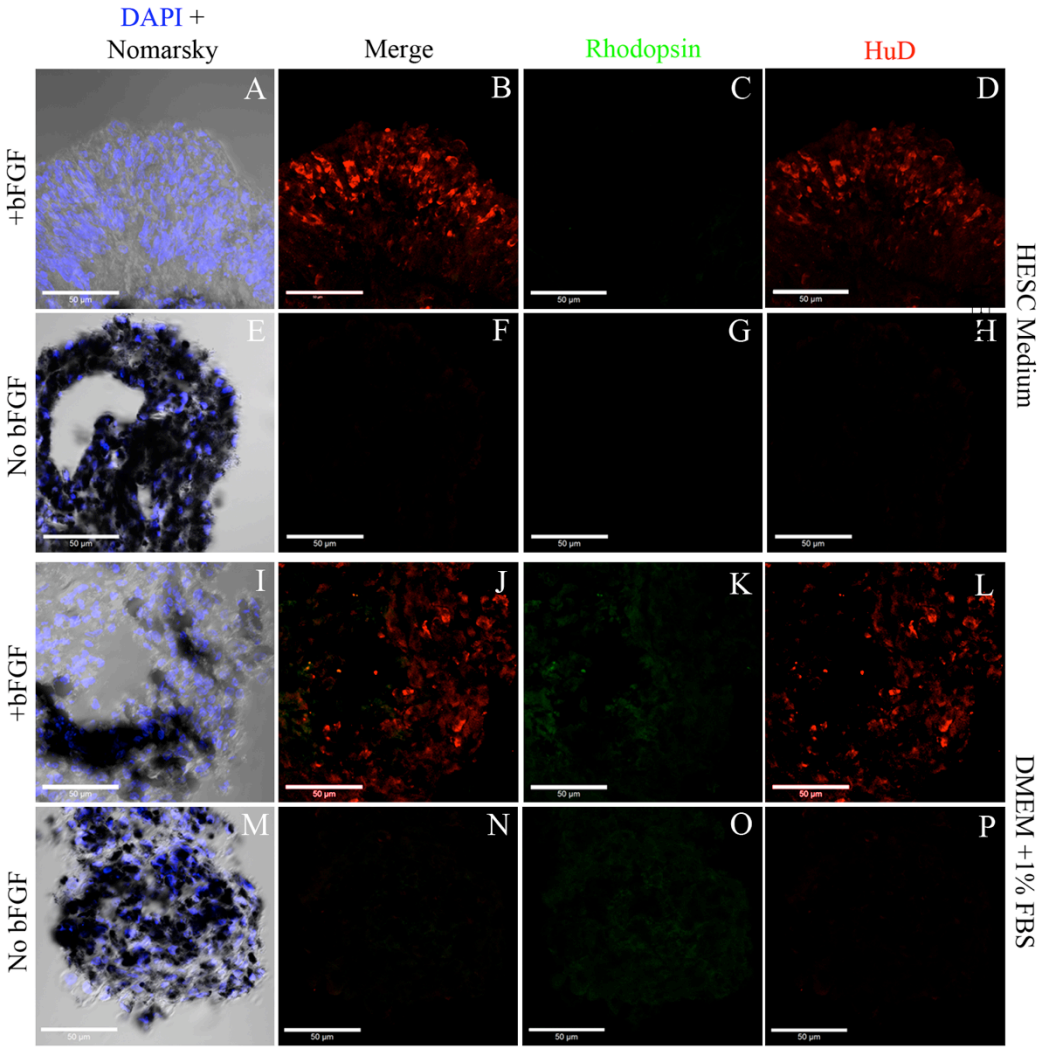


Fig. 3.29ii

Chick RPE HH26 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 2, Some transdifferentiation - Retinal cell specific markers.

Both bFGF treated explants in either HESC or control media were observed to exhibit a relatively robust level of transdifferentiation. This is evidenced by the loss of pigmentation, and the formation of pseudo-neuroepithelial structures in bFGF treated explants (A, I), in addition to the expression of ganglion/amacrine cell specific marker HuD (D, L). However, no explant was observed to express photoreceptor marker, rhodopsin, regardless of which culture medium was employed (C, K). Untreated, negative controls retained the characteristically pigmented phenotype (E, M), which was negative for retinal cell specific markers HuD (H, P), and rhodopsin (G, O). Fluoresence digitally enhanced. HESC medium (A-H), DMEM/F12 + 1% FBS control medium (I-P). Scale bars: 50uM.

Despite the fact that a de-pigmented, neuroepithelial-like structure was observed on the surface of one bFGF treated HH26 RPE explant cultured in HESC medium (Fig. 3.28iA 3.28iiA), whereas the untreated negative control retained a heavily pigmented phenotype (Fig. 3.28iE; 3.28iiE), no retinal progenitor markers were detected in this region (Fig. 3.28iC, D). Pax6 (Fig. 3.28iD) and Sox2 (Fig. 3.28iC) were both absent from this explant, which was also the case in the untreated, negative control (Fig. 3.28iG, H). Similarly, No evidence for transdifferentiation was apparent in the explant cultured in control medium, with no expression of Pax6 (Fig. 3.28iL, P) or Sox2 (Fig. 3.27iK, O) observed, regardless of treatment with bFGF.

Despite this, some de-pigmentation in response to bFGF was apparent (Fig. 3.28iI; 3.28iiI) when comparing this explant with the untreated negative control (Fig. 3.28iM; 3.28iiM). The fact that no HuD (Fig. 3.28iiD, H, L, P) or rhodopsin (Fig. 3.28iiC, G, K, O) expression was observed in any explant, regardless of the treatment with bFGF, would support a lack of transdifferentiation.

In contrast, RPE explants from another embryo stage HH26, did exhibit some evidence for transdifferentiation, in both HESC and control media (Fig. 3.29i; 2.29ii). A bFGF treated HH26 RPE explant exhibited a non-pigmented, neuroepithelium (Fig. 3.29iA; 3.29iiA), which was absent from the untreated explant (Fig. 3.29iE; 2.29iiE). This region was observed to express both Sox2 (Fig. 3.29iC) and Pax6 (Fig. 3.29iD), which co-localised in a number of cells (Fig. 3.29iB) indicating the presence of retinal progenitors. Pax6 and Sox2 were both absent from untreated negative controls (Fig. 3.29iG, H). A similar expression pattern was also observed in the bFGF treated explant cultured in control medium, which displayed a de-pigmented neuroepithelial-like region (Fig. 3.29iI; 3.29iiI), which was not present in the negative control (Fig. 3.29iM; 3.29iiM). This region was observed to weakly express Pax6 in a few nuclei (Fig. 3.29iL), as well as Sox2 (Fig. 3.29iK) in the majority of the nuclei. Both of these markers were again absent from the negative controls (Fig. 3.29iO, P).

Both bFGF treated explants in HESC and control media displayed some expression of HuD, with more cells expressing the protein in the HESC medium explant (Fig. 3.29iiD) than

that it control medium (Fig. 3.29iiLi). No rhodopsin expression was observed in any explants, in both culture media, regardless of the treatment with bFGF or not (Fig. 3.29iiC, G, K, O), and no HuD expression was observed in negative controls (Fig. 3.29iiH, P).

Once again, HH27 chick RPE explants exhibited a variable capacity for transdifferentiation in response to bFGF (Fig. 3.30i; 3.30ii; 3.31i; 3.31ii), perhaps with a more limited capacity than RPE at stage HH25 and HH26. One HH27 RPE explant did not display robust characteristics of transdifferentiation, other than somewhat de-pigmented, neuroepithelial-like structures in some regions at the surface of the explant in both HESC and control media in response to bFGF (Fig. 3.30iA, I; 3.30iiA, I). Despite this, the majority of these bFGF treated explants retained heavy pigmentation (Fig. 3.30iA, I; 3.30iiA, I) akin to the untreated negative controls (Fig. 3.30iE, M; 3.30iiE, M), which did not exhibit neuroepithelial regions. No retinal progenitor markers, including Sox2 (Fig. 3.30iC, G, K, O) and Pax6 (Fig. 3.30iD, H, L, P) were observed in bFGF treated, or untreated explants. Similarly, rhodopsin expression was absent from all explants in this experiment (Fig. 3.30iiC, G, K, O), which was also found to be the case for HuD expression (Fig. 3.30iiD, H, P), except for bFGF treated RPE in control medium, which expressed HuD in a couple of isolated cells within the neuroepithelial-like region (Fig. 3.30iiP).

A repeat of this experiment using RPE explants isolated from another embryo showed that some RPE cells at HH27 do retain the capacity to transdifferentiate in response to bFGF treatment (Fig. 3.31i; 3.31ii). No evidence for transdifferentiation was observed in the explant cultured in control medium at HH27, regardless of treatment with bFGF (Fig. 3.31iE-H 3.31iiE-H), with the bFGF treated explant largely retaining its pigmentation, with no neuroepithelial structures present (Fig. 3.31iI; 3.31iiI). Consistent with this phenotype was a lack of retinal marker expression, including: Sox2 (Fig. 3.31iK, O), Pax6 (Fig. 3.31iL, P), rhodopsin (Fig. 3.31iiK, O), or HuD (Fig. 3.31iiL, P). In contrast, bFGF treated RPE in HESC medium displayed classical evidence for transdifferentiation, with a non-pigmented neuroepithelium having formed (Fig. 3.31iA, Q) in comparison to the pigmented RPE phenotype maintained in the negative control (Fig. 3.31iE, U).

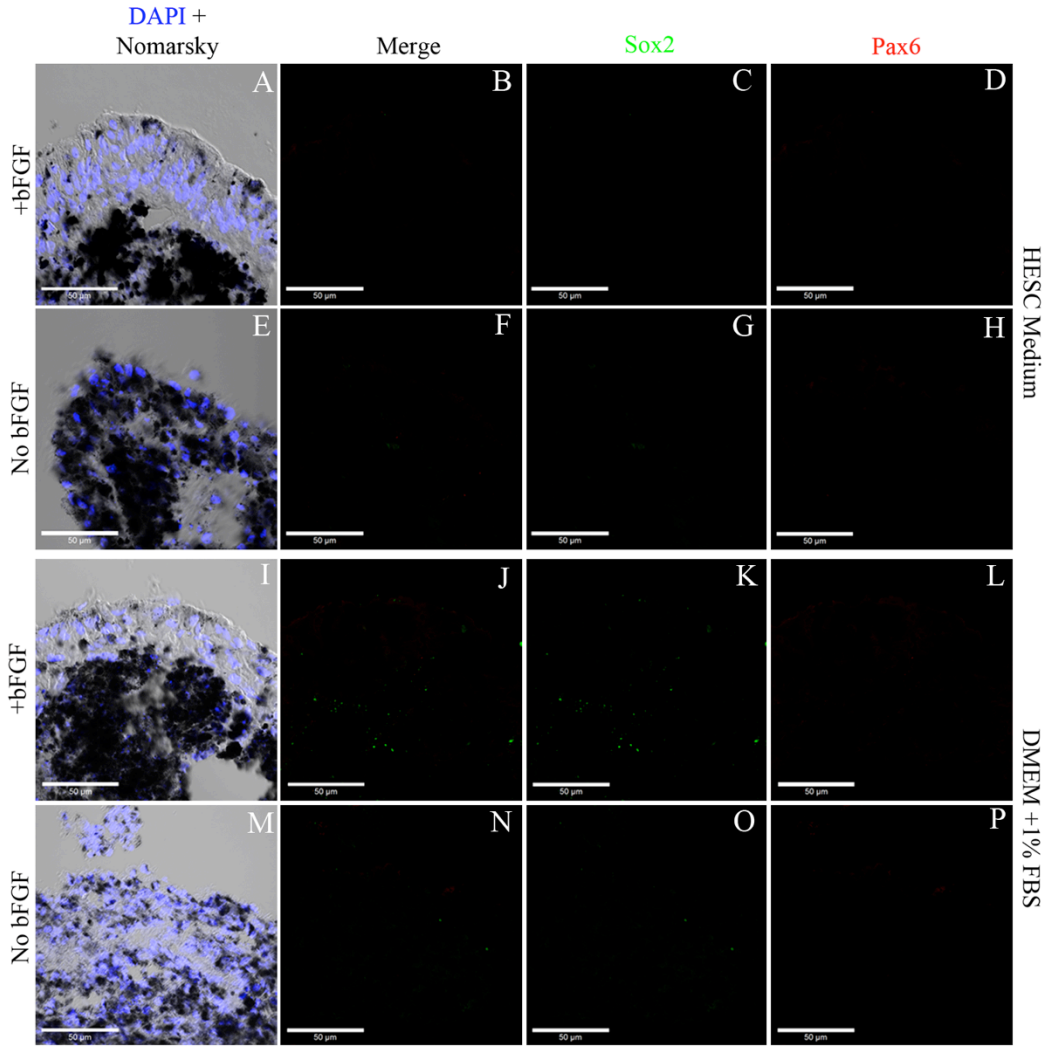


Fig. 3.30i

Chick RPE HH27 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 1, Limited evidence for transdifferentiaition - Retinal progenitor markers.

RPE explants at HH27 continue to exhibit variable capacity to transdifferentiate in response to bFGF in either medium. This embryo did not exhibit robust transdifferentiation in response to bFGF, despite the fact that some limited de-pigmentation, and formation of neuroepithelial-like strcutures at the surface of +bFGF explants were observed in both media (A, I). This is consistent with the reports that chick RPE loses the capacity to transdifferentiate during development. These de-pigmented, neuroepithelium-like structures were largely negative for retinal progenitor markers, including: Pax6 (D, L) and Sox2 (C, K). -bFGF controls retained pigmentation and did not express retinal progenitor markers (G, H, O, P) in either culture medium (E, M). HESC medium (A-H), DMEM/F12 + 1% FBS control medium. Scale bars: 50um.

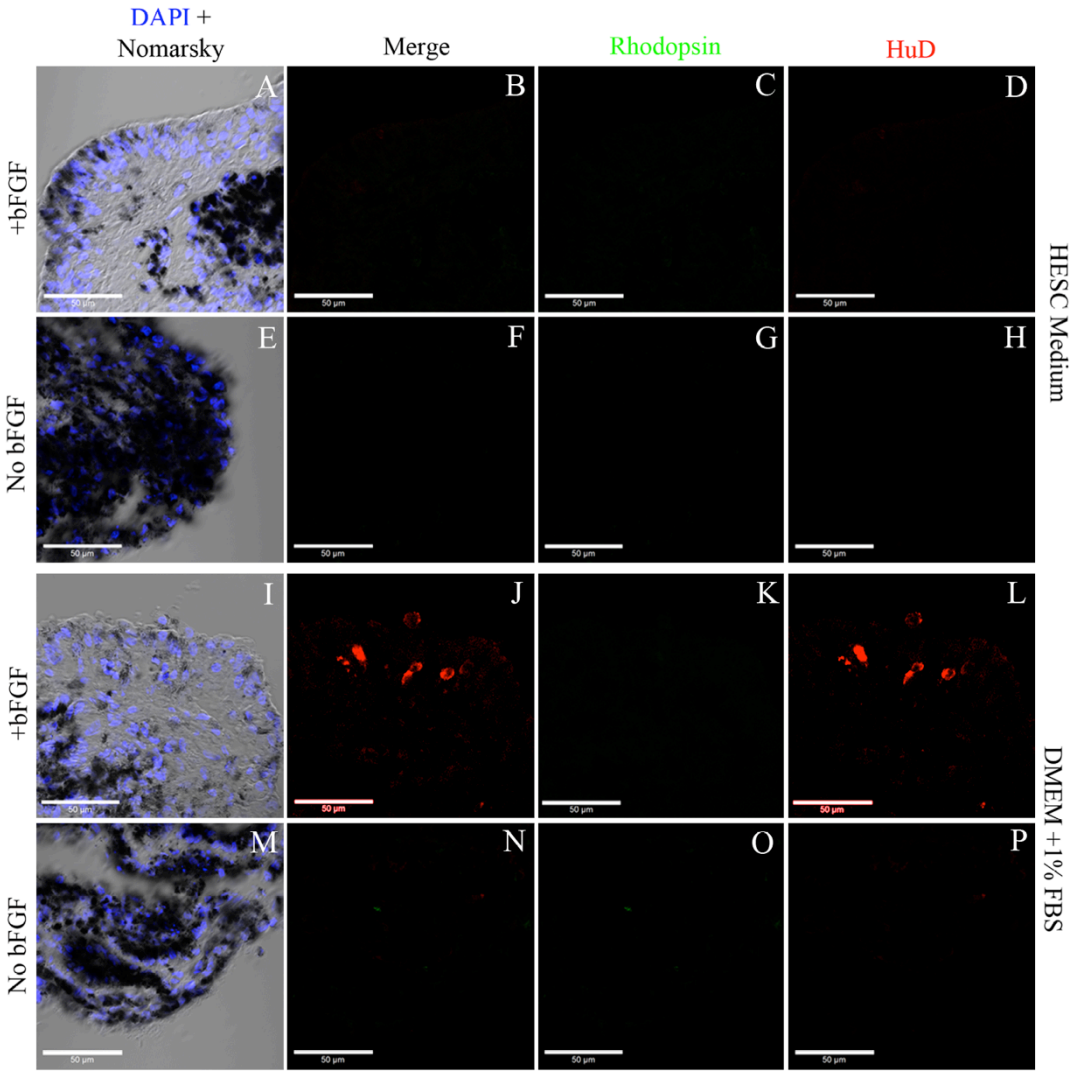


Fig. 3.30ii
Chick RPE HH27 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 1, Limited evidence for transdifferentiaition - Retinal specific markers.
These explants did not exhibit robust evidence for bFGF-induced transdifferentiation. Chick RPE explants HH27 treated with bFGF did not express retinal progenitor markers but did exhibit some limited HuD expression, in this instance in an explant cultured in control medium (L), in a region which somewhat resembles a de-pigmented, retinal neuroepithelium. The bFGF treated explant grown in HESC medium did not exhibit any HuD expression, despite exhibiting a similar structure in respone to bFGF (D). Neither HESC medium or Control medium cultured explants exhibited rhodopsin expression in repsonse to bFGF (C, K). Untreated explants did not exhibit any evidence for transdifferentiation, including no expression of retinal specific markers HuD (H, P), or rhodopsin (G, O), and retention of robust pigmentation (E, M). HESC medium (A-H), DMEM/F12 + 1% FBS control medium (I-P). Scale bars: 50uM.

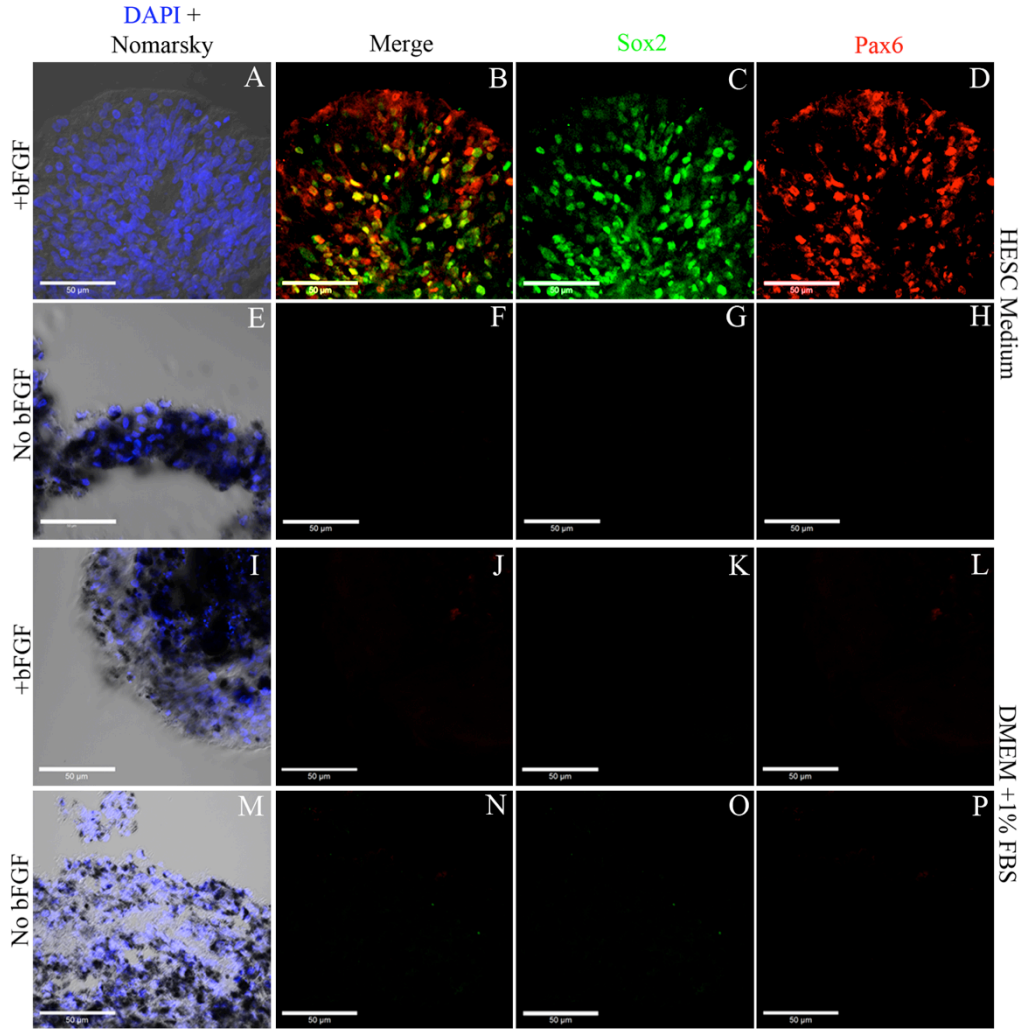


Fig. 3.31i

Chick RPE HH27 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 2, Limited transdifferentiation - Retinal progenitor markers.

The RPE from this embryo did exhibit some evidence for transdifferentiation at HH27. The RPE explant cultured in HESC medium exhibited a neuroepithelial region which was positive for Pax6 (D) and Sox2 (C) when treated with bFGF. Many of these cells were observed to express both markers, which suggests the presence of retinal progenitor cells. The +bFGF, control medium explant did not exhibit transdifferentiation, and retained a pigmented phenotype (I), which was negative for retinal progenitor marker expression (J-L). No negative controls exhibited retinal marker expressed and retained their pigmented RPE phenotype (E-H, M-P). Fluorescence digitally enhanced. HESC medium (A-H), DMEM/F12 + 1% FBS control medium (I-P). Scale bars: 50um.

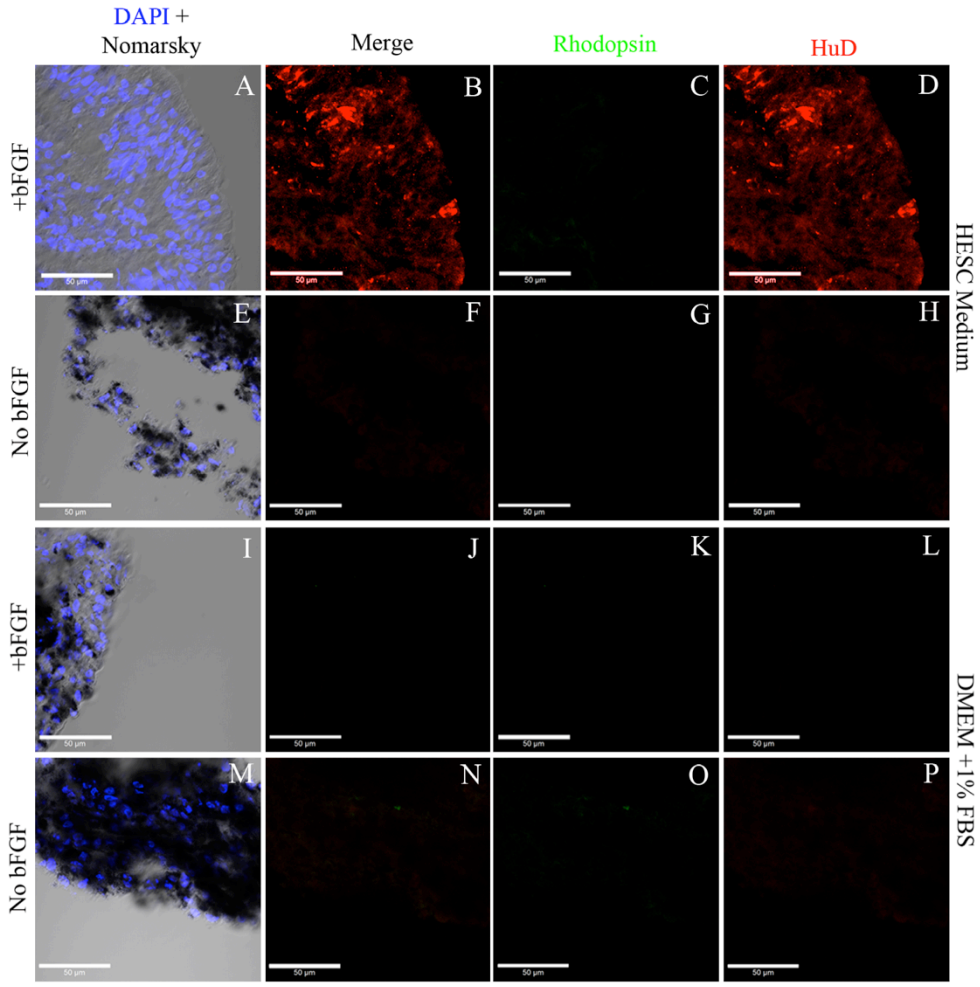


Fig. 3.31ii
Chick RPE HH27 +/- bFGF 7 days. HESC medium and DMEM/F12 + 1% FBS - Embryo 2, Limited transdifferentiation - Retinal specific markers.
Consistent with the expression of retinal progenitor markers in the bFGF treated explant cultured in HESC medium, this explant was also observed to express amacrine/Ganglion cell marker HuD (D) in a non-pigmented, neuroepithelial region of the explant after 7 days in culture. This region was however observed to be negative for photoreceptor marker, rhodopsin (C). The bFGF treated explant grown in control medium did not exhibit any evidence for transdifferentiation in response to bFGF treatment and was observed to retain robust pigmentation (I), in addition to the absence of retinal markers: HuD (L) or rhodopsin (K), in a similar manner to untreated negative controls cultured in both HESC (E, G, H) and control (M, O, P), which also retained the pigmented RPE phenotype. HESC medium (A-H), DMEM/F12 + 1% FBS control medium (I-P). Fluorescence digitally enhanced. Scale bars: 50uM.

This neuroepithelium contained a large number of cells expressing both Pax6 (Fig. 3.31iD, H) and Sox2 (Fig. 3.31iC, G), indicating the presence of retinal progenitor cells (Fig. 3.31iB). Neither of these transcription factors were expressed in the untreated negative control (Fig. 3.31iG, H). Additionally, this neuroepithelium was observed to express a limited amount of HuD, with no particular localization being evident, as a result of the low number of positive cells (Fig. 3.31iiD). No HuD expression was present in untreated RPE as expected (Fig. 3.31iiH).

Despite the fact that immunohistochemical data suggests that chick RPE explants do retain some capacity for transdifferentiation, topical observation of the explants after 7 days in culture does not necessarily show this very clearly (Fig. 3.32). Chick RPE, HH24 treated with bFGF clearly displays large areas of de-pigmentation, with the formation of low optical density neuroepithelial loops (Fig. 3.32A, C yellow arrows) in both HESC medium and control (DMEM/F12+1% FBS) medium. However, the untreated negative controls retained a heavily pigmented phenotype without any neuroepithelial loops (Fig. 3.32B, D). By HH25, bFGF treated explants do display some regions which appear to have lost pigmentation which are protruding from more intensely pigmented areas which are likely to have retained the RPE phenotype (Fig. 3.32E, G), however, these are less clear than at HH24. Similarly, at HH26, explants treated with bFGF in both media do exhibit some limited de-pigmentation, but lack the formation of any obvious, large neuroepithelial structures despite some limited de-pigmentation (Fig. 3.32I, K). Chick RPE explants stage HH27 show even less de-pigmentation in response to bFGF, in both culture media (Fig. 3.32M, O), with only some cells on the surface of the explant exhibiting some lighter pigmentation, however, no neuroepithelial loops are obvious in these cultures, which would suggest that no transdifferentiation has taken place akin to that observed at HH24 (Fig. 3.32A, C). No evidence of de-pigmentation or transdifferentiation was observed in any of the untreated negative controls in either culture media at any stage, where explants retained a heavily pigmented phenotype (Fig. 3.32 B, D, F, H, J, L, O, P).

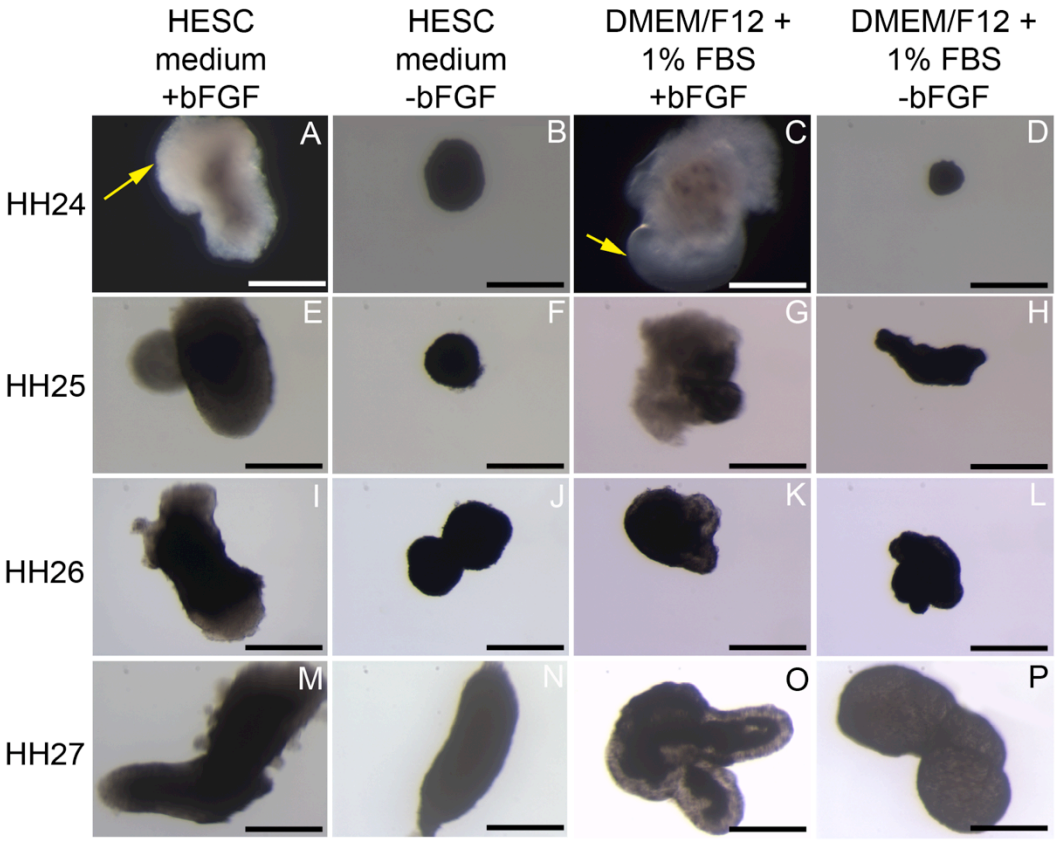


Fig. 3.32
Topical images of chick RPE aggregates at different developmental stages +/- bFGF 7 days. DMEM/F12 + 1% FBS and HESC medium.
HH24 explants treated with bFGF exhibited large regions of de-pigmented, neuro-epithelial loops with low optical density (A, C, yellow arrows).+bFGF HH25 explants did not exhibit as much de-pigmentation as HH24, however, some less pigmented protrusions were observed in both HESC and control media (E, G). By HH26, +bFGF explants only exhibted small, less-pigmented regions (I, K), and HH27, +bFGF explants exhibited even smaller protrusions, with the majority of the depigmented regions being localised to the surface of the aggregates (M, O). -bFGF controls retained intense pigmentation with no neuroepithelial protrusions at all stages of development (B, F, J, N, D, H, L, P). Scale bars: 200um.

3.5.4 Discussion:

Post-dissection controls demonstrated that RPE explants were pure following dispase treatment, and that no retinal markers were up-regulated by the dissection procedure. However, Pax6 expression was observed in all RPE cells until HH25, where no Pax6 expression was observed. This serves to highlight the variability of Pax6 expression across the RPE monolayer during development, as discussed earlier.

Surprisingly, this investigation found that RPE explants after stage HH25 are able to undergo transdifferentiation, contrary to previous reports that suggest embryonic chicken RPE can only transdifferentiate in response to bFGF up until this stage (Pittack et al., 1997, Park and Hollenberg, 1991, Park and Hollenberg, 1989, Park and Hollenberg, 1993, Pittack et al., 1991, Sakami et al., 2008). Additionally, it would appear that the ability for RPE to undergo transdifferentiation towards neural retina diminishes with the development of the RPE at later stages and this was not dependent upon the culture medium. However, without many more biological repeats of the experiment, this cannot be currently quantified. Needless to say, no qualitative trend was apparent for either medium, with both exhibiting variable potential for transdifferentiation. Some explants at later stages did not display robust evidence for transdifferentiation, including only limited retinal marker expression, whereas others did. This included the formation of both neuroepithelial, and pseudo-neuroepithelial structures, which often expressed a variable amount of retinal markers in some cells. Despite the disparity in the potential for transdifferentiation at different developmental stages, bFGF treated RPE at all stages did appear to exhibit at least a limited response to the growth factor, through loss of pigmentation at the surface of the explant. Interestingly, no RPE explants stage HH26 and beyond displayed the expression of rhodopsin, which would tend to suggest that the capacity for transdifferentiation of the RPE is diminished at these stages when compared with less mature RPE at HH24/25. If less robust transdifferentiation is observed, the likelihood is that the resulting retinal cells cannot develop as well over the period in culture.

The source of this variability in the capacity to transdifferentiate is unclear, however, there are a number of possible explanations. Several of the previous studies appeared to analyse whether or not transdifferentiation had occurred at later stages through topical analysis only (Sakami et al., 2008, Pittack et al., 1991). Transdifferentiation observed to take place at later stages is less obvious in culture because of the absence of obvious neuroepithelial loops, which are clearly present in transdifferentiated RPE from earlier stages, for example HH24. This may mean that later stage explants had been incorrectly categorised as not having undergone transdifferentiation, when in fact transdifferentiation, in the form of retinal gene expression, had taken place to a degree.

In addition, other differences between the present study, and previous investigations exist, for example, given the intricate difficulties in dissection of the RPE tissue from embryonic eyes, it is possible that different regions of the RPE monolayer have difference capacities for transdifferentiation and that different studies may have isolated slightly different regions. This could explain the discrepancies between the different datasets. It is perhaps unsurprising that RPE cells would appear to lose the ability to transdifferentiate in a more gradual manner, rather than at a stark stage of development, given the fact that RPE monolayers are thought to develop in a non-uniform manner. One would therefore expect any capacity to transdifferentiate to be lost in a similarly gradual manner.

Difficulties in manipulation of explanted RPE sheets also make it difficult to control the size of each explant, which further adds to the variability of the system. This is particularly important given the fact that RPE cells at these stages have been reported to express activin, a signaling factor which represses the effects of bFGF on RPE cells and therefore restricts the capacity for transdifferentiation (Sakami et al., 2008). It is possible that different sizes of RPE explant could therefore lead to different concentrations of inhibitory activin being present, which would potentially vary the capacity for transdifferentiation. However, given the likely small differences in activin concentration, in comparison to the over-whelming, high concentration of bFGF treatment, it is unreasonable to suggest that activin from the RPE alone could account for variation.

Perhaps more important is the presence, or lack thereof, of mesenchymal tissue. RPE explants treated with dispase to remove this tissue has shown that the presence of mesenchyme is not necessary for transdifferentiation of the RPE as one would expect given its role in specification and augmentation of the RPE phenotype (Fuhrmann et al., 2000b, Muller et al., 2007). Previous studies have explanted RPE cells with a significant amount of mesenchyme still attached to the RPE monolayer. This is significant because the extra-ocular mesenchyme has been shown to express much higher concentrations of activin, approximately 100-fold higher than that in the RPE. It is possible that this contaminating material could act to antagonise bFGF treatment much more significantly than activin autocrine signaling from the RPE itself. In this investigation, even without dispase treatment, RPE tissue was only contaminated with a very small amount of mesenchymal tissue when qualitatively compared to those in previous reports. This could perhaps extend the developmental window for capacity for transdifferentiation because of a lower concentration of activin signaling adjacent to the RPE, and therefore less inhibition. This is a particularly compelling hypothesis given the fact that pharmacological inhibition of activin signaling has been reported to extend this window of competence by up to 2 days (Sakami et al., 2008), however, given that some evidence for transdifferentiation at later stages (HH27) was observed in non-dispase treated explants, this seems unlikely.

Another study has observed similarly variable result in the ability to transdifferentiate at different developmental stages, using a different culture system. RPE could transdifferentiate up to stage HH26, with no explants transdifferentiating after this stage (Coulombre, 1981). The authors also observed a gradual decline in the capacity for transdifferentiation, which correlated with the development of the RPE. Different developmental cut-off points for transdifferentiation could easily be accounted for in the dynamic nature of the developmental process. This is certain to lead to variability in results.

Interestingly, the gradual loss of the capacity for transdifferentiation after stage HH24 appears to correlate with a loss in the expression of Pax6 discussed previously. This is interesting given that Pax6 is regarded as the master regulator of transdifferentiation, and an

increase in expression is required for transdifferentiation (Spence et al., 2007b, Azuma et al., 2005a). The presence of Pax6 has also been suggested as a critical factor for the onset of transdifferentiation in regenerating newt RPE cells (Kuriyama et al., 2009b). Here it is postulated that RPE cells must first start to express Pax6 during the onset of RPE transdifferentiation, via an altered tissue interaction with the choroid. This effect is observed to be reversible, with bFGF signaling required for maintenance and the progression of transdifferentiation. Therefore, given the fact that RPE transdifferentiation is restricted to embryonic stages in the chick, it is possible that this is because the presence of Pax6 is required for exogenous bFGF treatment to be effective and trigger progression of transdifferentiation. Once Pax6 expression in the RPE has been down-regulated during development, bFGF can no longer be effective as chicken RPE cells are unable to re-up-regulate Pax6 once it is lost. Consistent with this idea is the fact that over-expression of Pax6 *in vivo* is able to cause RPE to undergo transdifferentiation at developmental stages much later than bFGF is able to (Azuma et al., 2005a). Furthermore, activin signaling, which is know to restrict the ability of RPE cells to undergo transdifferentiation in response to bFGF treatment, has been shown to inhibit the expression of Pax6 (Fuhrmann et al., 2000b), which may be the mechanism by which it is able to do so.

If Pax6 expression is responsible for the capacity for transdifferentiation, this could therefore explain the variability observed in RPE explants from the same stage in undergoing transdifferentiation. Pax6 expression was observed to be down-regulated at different times in a regional manner, and therefore, some explanted sheets might retain expression of Pax6, and therefore the capacity for transdifferentiation, while others do not. This would also account for the variation in the ability for transdifferentiation seen in separate explants from the same eye (in different media) as maintenance of Pax6 expression may vary. Note that there does not appear to be a bias for the capacity of transdifferentiation which is dependent on the media used, however, in order to statistically confirm this, many more explants would need to be tested for quantification.

Explants exhibiting small amounts of transdifferentiation at later stages could imply that transdifferentiation at later stages is delayed in comparison to that of early stages. This idea is supported by the fact that no rhodopsin staining was observed in any of these explants. However, given the apparent involvement of Pax6 expression, it is possible that these explants only retained small clusters of Pax6 positive cells, which were unable to form fully developed ectopic retinas.

Interestingly, some untreated explants at earlier stages of development at HH25 and below were also observed to retain the expression of Pax6 after 7 days in culture, which contrasted with most untreated explants, which were observed to down-regulate expression of the transcription factor, possibly as a feature of normal maturation. It remains unclear why some untreated explants retained Pax6 expression, and others did not, however, this could reflect the variable expression of Pax6 across the RPE sheet discussed earlier. It is likely that Pax6 expression was only observed in earlier transplants given that Pax6 is likely to be retained in more of the developing RPE monolayer, than at later stages when it begins to be down regulated across the monolayer. It remains to be seen whether or not these Pax6 positive RPE cells would still retain the capacity for transdifferentiation if treated with bFGF, however, this does demonstrate that Pax6 expression alone is insufficient to induce transdifferentiation, given that these untreated explants retained their characteristically, pigmented RPE phenotype. It is possible that a threshold level of Pax6 is required for the initiation of transdifferentiation, and/or additional factors, such as bFGF, may be required to regulate additional transcription factors, such as Sox2 (Ma et al., 2009).

Some RPE explants treated with bFGF did not appear to undergo transdifferentiation at later stages, however, they did appear to still respond to bFGF, exhibiting some areas of de-pigmentation. This once again supports the idea that a down-regulation in the expression of the bFGF receptor is not responsible for the loss in capacity for transdifferentiation. This de-pigmentation may suggest that bFGF activates several signaling pathways with specific functions. It may be that in this instance, the neural differentiation branch of the pathway is inhibited, but at least a portion of the RPE specification pathway is inhibited. The loss in

pigmentation would perhaps suggest the down-regulation of Mitf expression in the RPE, which is responsible for pigmentation (Shibahara et al., 2000).

In conclusion, the loss in the capacity for transdifferentiation is a variable process that occurs gradually after HH24, and the maintenance of Pax6 expression could be crucial for the on-going retention of the potential for transdifferentiation of RPE cells. In addition, HESC medium does not appear to contain any products which can inhibit, or potentiate, the ability for transdifferentiation at different stages of RPE development. Therefore, HESC medium is suitable for on-going studies of transdifferentiation in both chicken and human models of transdifferentiation. Future studies will focus on signaling pathways which are able to modulate the expression of Pax6, and may therefore be candidates for the restriction of the ability for transdifferentiation. Additionally, it will be necessary to analyse the changes in expression after HH24 in order to identify new candidates that may restrict bFGF-mediated transdifferentiation.

Chapter 4 –

Investigation into signaling pathways which may restrict the capacity for transdifferentiation of the RPE

4.0 Introduction

It is unclear why embryonic chick RPE is observed to lose the capacity to transdifferentiate in response to bFGF with the progression of development. If the mechanisms responsible for the limitation in the capacity for transdifferentiation can be elucidated, it may be possible to manipulate them in order to re-activate the potential for transdifferentiation in more developed RPE cells, which would not normally undergo the phenomenon in response to bFGF-treatment.

Pax6 appears to be critical for the retained capacity for transdifferentiation (Spence et al., 2007b, Azuma et al., 2005a, Arresta et al., 2005, Sakami et al., 2008), in addition to induction of the phenomenon, in a number of models of the phenomenon. In the *xenopus* model, it has been suggested that bFGF does not actually induce the initial expression of Pax6 itself, however, it is required for the maintenance in expression of the transcription factor. This appears to be crucial for full regeneration of the retina to take place (Kuriyama et al., 2009a). It is suggested that RPE cells must first migrate away from the choroid before expression of Pax6 becomes active in the RPE, and that this is a reversible process that was shown to be independent of bFGF signaling. Furthermore, it was shown that once Pax6 is expressed in the RPE, bFGF can then act on these Pax6 positive cells and subsequently drive them towards a multi-potent neuroblastic state. Mammalian and embryonic chicken RPE do not share the same regenerative capabilities as their amphibian counterparts, and therefore, it is likely that embryonic chicken RPE cells may not have the capacity to re-express Pax6 (the first proposed stage of RPE transdifferentiation) via an altered interaction with the extracellular matrix, and/or other cells. Therefore, they may require the expression of Pax6 to be maintained in RPE cells, so that bFGF signaling can activate the second proposed stage of transdifferentiation, the differentiation of RPE cells into multi-potent retinal neuroblasts. bFGF most-likely promotes transdifferentiation through further up-regulation of Pax6, which in turn down-regulates RPE-specific transcription factors

such as Mitf and Otx2 (Spence et al., 2007b, Avdonin et al., 2008)., and up-regulate retinal markers, such as Sox2 (Ma et al., 2009), which are important for retinal progenitor formation.

If the maintenance of Pax6 expression is the limiting factor for the ability of RPE cells to transdifferentiate, it is reasonable to suggest that a signaling mechanism, or possibly a number signaling mechanisms, exist that are responsible for the down-regulation of Pax6 in the developing RPE. This may terminally augment the RPE phenotype and prevent it from responding to autocrine signals involved in the development of the rest of the eye. It is possible that without the down-regulation of Pax6 and subsequent loss in potential for transdifferentiation, normal signaling in the eye at later developmental stages might still be able to induce transdifferentiation of the RPE. This would obviously have detrimental effects on the proper development of the eye, and may result in syndromes such as micropthalmia (Araki et al., 2002, Mochii et al., 1998b).

Activin-like signals help to specify the fate of RPE cells in the adjacent, outer-layer of the optic cup, via a down-regulation of neural retinal transcription factors such as Pax6 and Chx10, possibly through augmentation of RPE transcription factors such as Mitf (Nguyen and Arnheiter, 2000, Fuhrmann et al., 2000b). Consistent with its role in augmenting the RPE phenotype, exogenous addition of activin A to cultures of HH23/24 embryonic chick RPE explants treated with exogenous bFGF, employing a similar non-adherent culture method as the one used in this investigation, resulted in the inhibition of transdifferentiation in response to bFGF (Sakami et al., 2008). Additionally, it was reported that subsequent pharmacological inhibition of the activin signaling pathway was not only able to maintain the capacity for transdifferentiation of RPE towards neural retina, but it also reported a reversal of the loss of potential for transdifferentiation. In each case, the maximum extension of the window of sensitivity to bFGF was an additional 2 days of development i.e. up until E6 (HH29), with no further effect of the drug after this point.

As activin inhibition only appears to enhance the capacity for transdifferentiation by a limited amount, this would imply the action of other regulatory mechanisms that act in synergy with activin to augment to RPE phenotype. Bone morphogenetic proteins (BMP) and

sonic hedgehog (Shh) signaling pathways have both been shown to regulate the expression of Pax6 within the RPE and also induce transdifferentiation of the RPE upon inhibition during development (Zhang and Yang, 2001, Spence et al., 2004, Muller et al., 2007).

The expression of BMP signaling molecules is often associated with the development of the RPE, and as a consequence, BMP expression is often used as an RPE marker given the expression of BMP-family members in both developing and adult RPE cells (Mathura et al., 2000, Vogel-Hopker et al., 2000, Muller et al., 2007). RPE cells are also known to express different types of BMP-receptors throughout different stages of development (Belecky-Adams and Adler, 2001). BMPs are also expressed in the surface ectoderm, over-lying the optic cup, as well as in the extra-ocular mesenchyme which, as previously discussed, has been shown to be important in the specification of an RPE fate (Muller et al., 2007, Fuhrmann et al., 2000b, Belecky-Adams and Adler, 2001). In addition, BMPs are heavily implicated in the dorso-ventral patterning of the retina as a whole, primarily specifying a dorsal identity (Sasagawa et al., 2002, Murali et al., 2005, Adler and Belecky-Adams, 2002, Prada et al., 1992). The inhibition of BMP signaling through the application of a natural BMP-antagonist, such as noggin, has shown that BMP-signaling is crucial for the specification of the RPE following invagination of the optic cup (Muller et al., 2007, Adler and Belecky-Adams, 2002). Noggin treated embryos had regions where the RPE had not formed properly and appeared to have undergone transdifferentiation towards a neuroepithelial phenotype. This included the loss of pigmentation compared with controls and a down-regulation of characteristic RPE markers such as Otx2 and Mitf. The neuroepithelium was instead observed to be expressing markers like Rx and Chx10, which are normally associated with the developing retina (Muller et al., 2007). In addition, noggin application was also observed to up-regulate the expression of Pax6 in the RPE, which would indirectly suggest that BMP-signaling suppresses the expression of Pax6 under normal conditions *in vivo.* BMP-signaling has also been implicated in demarcating developmental boundaries in conjunction with an FGF-signaling family member, FGF8, in the development of the rostral prosencephalon during the development of the telencephalic and optic vesicles (Ohkubo et al., 2002). Additionally, BMP4-coated beads

implanted into the developing heads of chicken embryos have been shown to result of a down-regulation of FGF8.

BMP-family member, BMP2 has also been shown to antagonize the effects of FGF-4 during limb development via inhibition of the proliferation of mesenchymal cells (Niswander and Martin, 1993). Given that transdifferentiation of the RPE requires cells to de-differentiate and proliferate, this may be a mechanism by which BMP-family members regulate the ability to transdifferentiate. Intriguingly, BMP-signaling members have been reported to interact with extracellular matrix proteins, such as collagen, as well as proteoglycans like heparin (Reddi, 1995, Ruppert et al., 1996), a co-factor which is reported to be very important in the regulation of FGF-signaling (Caldwell et al., 2004, Caldwell and Svendsen, 1998, Carwile et al., 1998, Chai and Morris, 1999, Cirillo et al., 1990, Forsten-Williams et al., 2008, Furue et al., 2008, Giftochristos and David, 1988, Kim et al., 2003, Ornitz et al., 1992, Yayon et al., 1991, Yu et al., 2007). BMP-family members may interact with heparin in a manner which prevents FGF signaling from taking place.

Sonic hedgehog (Shh) signaling has also been shown to be important in the spatio-temporal patterning of the developing embryo, including the eye, and this regulation is equally as complex as BMP-signaling (Zhang and Yang, 2001, Yu et al., 2006, Wang et al., 2002, Spence et al., 2004, Perron et al., 2003, Ohkubo et al., 2002, Moshiri and Reh, 2004, Levine et al., 1997, Marigo, 2008, Marigo et al., 1996a, Marigo et al., 1996b). Shh is known to signal through a transmembrane receptor called Patched-1 (Ptc1), (Marigo et al., 1996a, Marigo et al., 1996c, Stone et al., 1996) which, upon activation, is able to up-regulate its own expression. As such, it is a good marker of active Shh signaling (Alcedo et al., 1996, van den Heuvel et al., 1996)(Ingram, Wicking et al. 2002). The fact that Ptc1 is known to be expressed in developing RPE cells indicates the role of Shh-signaling in RPE development (Zhang and Yang, 2001, Perron et al., 2003). In addition, it has been shown that the blockage in Shh signaling, either via the use of a pharmacological inhibitor such as cyclopamine, or through the transplantation of anti-Shh antibody secreting cells into the eye, results in a loss of RPE phenotype, including pigmentation and markers such as Mitf. This loss of RPE phenotype

correlates with the formation of an ectopic retina, which is positive for a number of retinal markers, including Chx10 and visinin (Perron et al., 2003, Zhang and Yang, 2001).

Blockage of Shh signaling resulted in an up-regulation in the expression of Pax6 in the retina (Perron et al., 2003, Zhang and Yang, 2001), as well as a decrease in Mitf expression in the RPE. In addition, ectopic expression of Shh led to a down-regulation of Pax6, with a subsequent increase in RPE marker Otx2 (Zhang and Yang, 2001). This possibly indicates that Shh may induce transdifferentiation of the RPE through modulation of Pax6 (Zhang and Yang, 2001). Therefore, could it be that Shh signaling is at least partly responsible for the limitation in the capacity to transdifferentiate in response to bFGF? Shh is reported to work synergistically with FGF2 in order to activate proliferation of the retinal stem cell population in the ciliary marginal zone (CMZ), and subsequent regeneration of the retina, in chicks, a phenomenon thought to be absent in humans (Spence et al., 2004). Similarly, in zebrafish, Shh is thought to interact with effectors of the FGF-pathway and thereby modulate expression patterns in the developing retina (Vinothkumar et al., 2008). This highlights the fact that Shh and FGF signaling are already known to converge during development. In addition, Shh has also been shown to have roles in the development of both the telencephalic and optic vesicles via interaction with FGF8 signaling pathways. This interaction is similar to that previously discussed for BMP-signaling, which may suggest a common mechanism for their observed regulation of RPE specification (Ohkubo et al., 2002, Crossley et al., 2001). If they do indeed exhibit control of the RPE phenotype in a similar manner, this shared factor has yet to be identified.

4.1 The effect of BMP signaling on the expression of Pax6 transcription factor, 'a master regulator of transdifferentiation.'

4.1.1 Introduction:

Explanted embryonic RPE sheets were isolated from the earliest stage of retinal development possible and treated with several members of the BMP signaling family in order to ascertain their capacity to modulate Pax6 expression, thought to be important for the eventual restriction of the capacity for transdifferentiation in response to bFGF. The evidence for the involvement of BMP-signaling in modulation of Pax6 expression in RPE cells is indirect, given that it is noggin-application, a BMP antagonist, which results in an up-regulation of Pax6 in RPE (Muller et al., 2007). This would suggest that BMP-application would likely have the reverse effect of down-regulation of Pax6 in RPE cells, however, given that noggin is able to inhibit the action of a number of different BMP family members (Lim et al., 2000, Adler and Belecky-Adams, 2002, Belecky-Adams and Adler, 2001), it may be possible that the noggin-dependent inhibition is specific to a particular BMP family member. Therefore, in addition to BMP4, two other BMP signaling factors, BMP5 and BMP7, were also tested in this assay.

4.1.2 Materials & Methods:

4.1.2.1 RPE cell isolation and culture:

Embryonic chicken RPE explants stage HH21 were isolated and purified using dispase treatment as described in chapter 2. These explants were transferred to Matrigel^{TM} (GIBCO) coated, tissue culture plastic and cultured under standard, adherent, RPE cell culture conditions, in DMEM/F12 +1% FBS (GIBCO). Standard chicken control medium (DMEM/F12 +1% FBS) was employed instead of HESC medium because HESC medium has

already been reported to contain a 'BMP-like activity,' which may have affected the results (Xu et al., 2005). Medium was supplemented with a single dose of 200ng/ml BMP4/5/7 on each day of culture until the experiment was terminated through fixation for 30 minutes in 4% PFA. The initial pilot experiment involved a 1day, RPE cell culture period +/-BMP4 only. Subsequent experiments employed a 2-day culture period supplemented with only one BMP family member at a time. Each RPE explant was divided into two, approximately equally-sized sheets to allow for one experimental, and one negative control condition which was untreated (See Fig. 4.1M for schematic explanation of culture procedure).

4.1.2.2 Immunohistochemistry, statistics and Image analysis:

Immunohistochemistry was performed as described in chapter 2.4. 16-bit gray scale images were produced from single-channel confocal microscope images using ImageJ software as described in chapter 2.5. It was then necessary to threshold the images in order to ensure that only positive labeling was taken into account in the mean pixel calculation. This was performed by thresholding the measurement to the most weakly stained cell in the image frame, which ensures that all other cells which have a higher intensity are subsequently measured in the mean pixel value calculation, and the background is not taken into account (Fig. 4.2 M) This method gives an overall average intensity for the fluorescence which is able to act as a representative measure of the gene expression level. The level of pigmentation was measured in a similar manner, using the same software program. However, owing to the fact that some areas of the RPE/neural retinal tissue in these assays contain pigment, and other do not, it was necessary to employ the use of a *'region of interest'* tool in order to select the area of tissue of interest (rather than thresholding). This tool was used to select smaller regions of interest within the sheet, which can be overlaid in the same area of multiple separate channels in the same image. This feature was used to analyse whether any correlation between the level of Pax6 expression, and pigmentation exists (Fig. 4.3 M). Several areas of both high and low pigmentation, and high a low expression of Pax6, were selected for analysis in each case. The

region of interest tool is more appropriate than thresholding for the measurement of pigmentation levels, as de-pigmented areas of tissue are often have a similar gray scale value as the background, which can make it difficult to select the areas of interest using the *threshold* tool. Statistical analysis was as described in chapter 2.11.

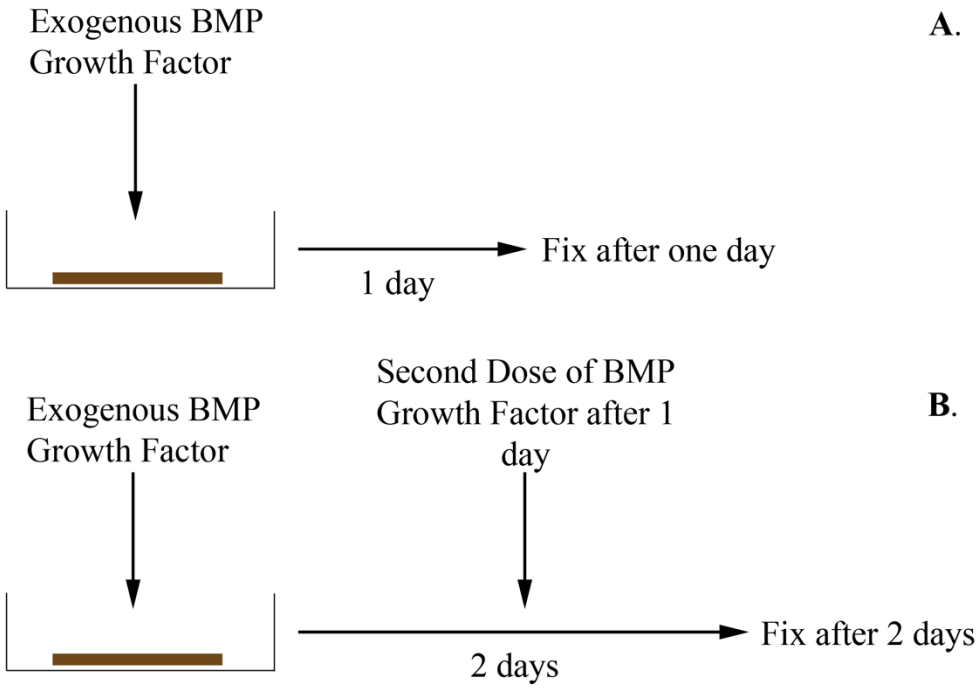


Fig. 4.1-M
Schematic of culture system for elucidating the effect of exogenously-added BMP growth factors on thr expression of Pax6.
RPE explants grown for 1 day in the presence of BMP4 for 1 day follow the culture system described in A. RPE explants which are treated with BMP growth factors for 2 days follow the culture system described in B.

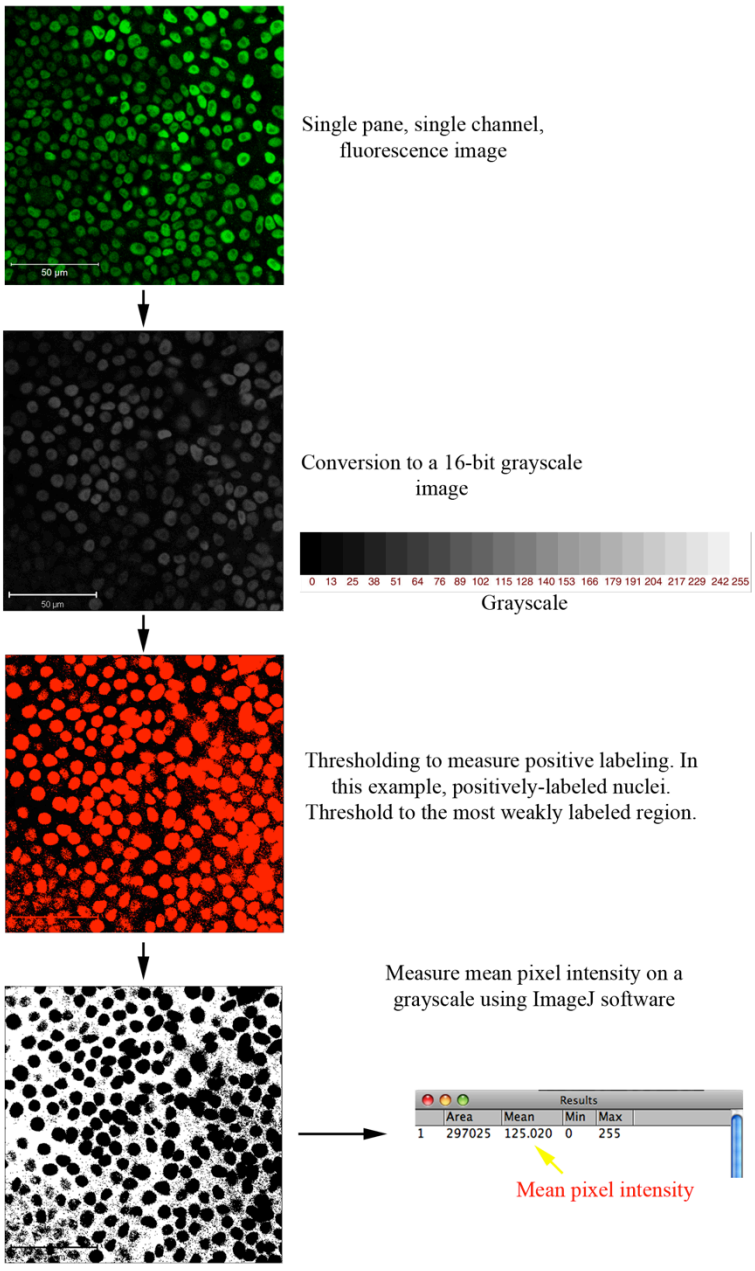


Fig. 4.2-M

Thresholding and measurement of mean pixel intensity of flurescence images in order to quantify relative expression.

In order to quantify the fluorescence intensity of a particular image, it was necessary to employ image analysis software to first create a grayscale image from the original, with each pixel being assigned a value from 0 (Black) to 255 (White) depending on it's intensity. This allows an average pixel intensity for the image to be calculated, which corresponds to the level of fluorescence in the original image. In order to only analyse those pixels which exhibited positive fluoresence, and not areas of the image which are not of interest, it was necessary to employ a threshold of pixel intensity which corresponded to the regions of interest. In order to include all positive pixel values, the threshold was set to the most weakly labeled pixel of the image by eye. The avearge pixel intensity was thus calculated from an average of all pixel values above this value.

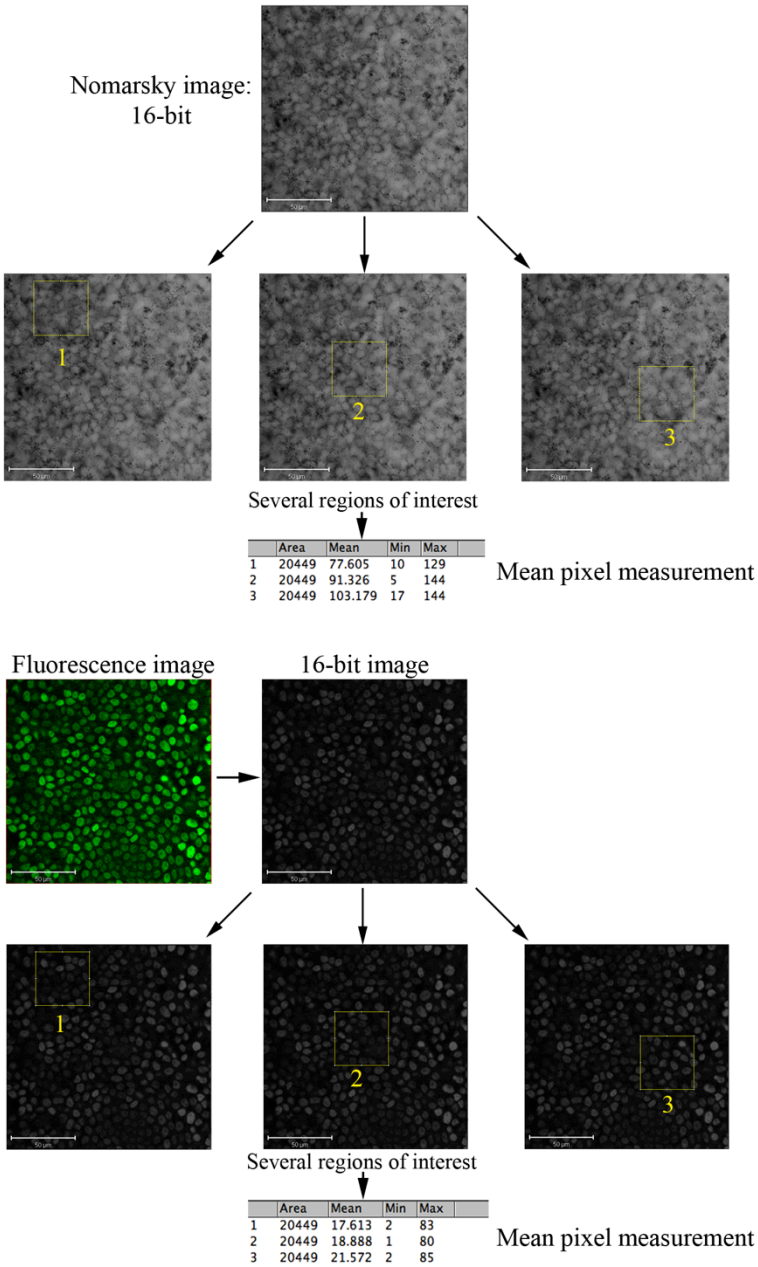


Fig. 4.3-M
Method of quantification of Pax6 expression level and corresponding level of pigmentation in multiple regions.
Several regions of the same area (numbered yellow boxes) with varying Pax6 levels were selected using a 'region of interest' tool. This allowed the level of pigmentation in the corresponding region to be measured. These values were then analysed to elucidate whether or not the level of Pax6 expression was linked to the level of pigmentation.

4.1.3 Results:

4.1.3.1 Effect of 1 day BMP4 treatment on Pax6 expression in explanted chick RPE HH21:

Lightly pigmented, Chick RPE sheets at a stage of HH21 (approximately E3.5) (n=3) readily adhered to Matrigel-coated, tissue culture plastic. Cells at the edge of the sheet appeared to lose their characteristic, pigmented, epithelial cell morphology, and began to migrate outwards, away from the centre of the explant in both culture conditions (Fig. 4.1). Migrating cells exhibited a fibroblastic phenotype and lost their characteristic pigmentation (data not shown).

Pax6 expression was maintained in both untreated, and BMP4-treated, RPE cell explants (Fig. 4.1A & 4.1B respectively). No clear differences in the level of Pax6 expression between treated and untreated was apparent. Similarly, no clear difference in the level of pigmentation of these explants was apparent (Fig. 4.1C, D).

4.1.3.2 Effect of 2 day BMP4 treatment on Pax6 expression in explanted chick RPE HH21:

Pax6 was expressed in the nuclei of both untreated and BMP4 treated explants (n=3) (Fig. 4.2A, E & B, F respectively). However, unexpectedly, the level of Pax6 expression was not consistent across the cells of the RPE monolayer with some cells expressing Pax6 at a high level, with others having a very low, barely detectable expression of the transcription factor (Fig. 4.2A, B yellow arrows). It was initially suspected that this may have resulted from the RPE sheet attaching to the dish in a ruffled manner, which caused different cells to lie in different visual planes, thus affecting the apparent level of Pax6 labeling. However, this was found not to be the case, as confirmed by a consistent pattern of Pax6 expression observed through images taken in a number of visual planes. Additionally, nuclear counterstaining with DAPI showed that these cells do in fact lie in the same plane (data not shown).

There did not appear to be a specific, observable pattern for the location of the regions of low Pax6 expression within the cultured explant. The localization was observed to

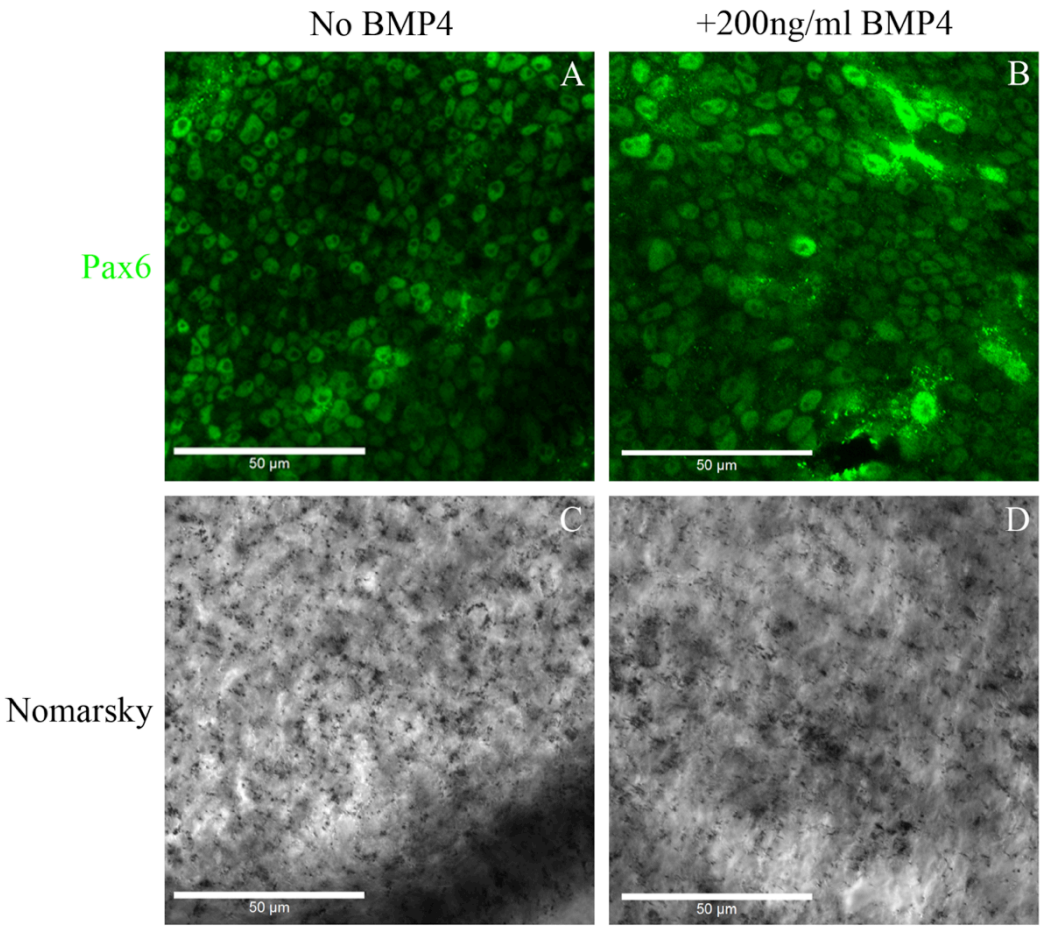


Fig. 4.1
Chick RPE explant HH21 on matrigel +/-BMP4 (200ng/ml) for 1 day.
Exogenous treatement of chick RPE explants with BMP4 does not down-regulate Pax6 expression in culture (B) when compred with untreated controls (A). Treatment with BMP4 does not appear to increase the level of pigmentation of the RPE (D) when compared with untreated controls (C). Pax6 (green) A, B. Nomarsky C, D. +BMP4 (B, D), -BMP4 (A, C). Scale bar - 50uM)

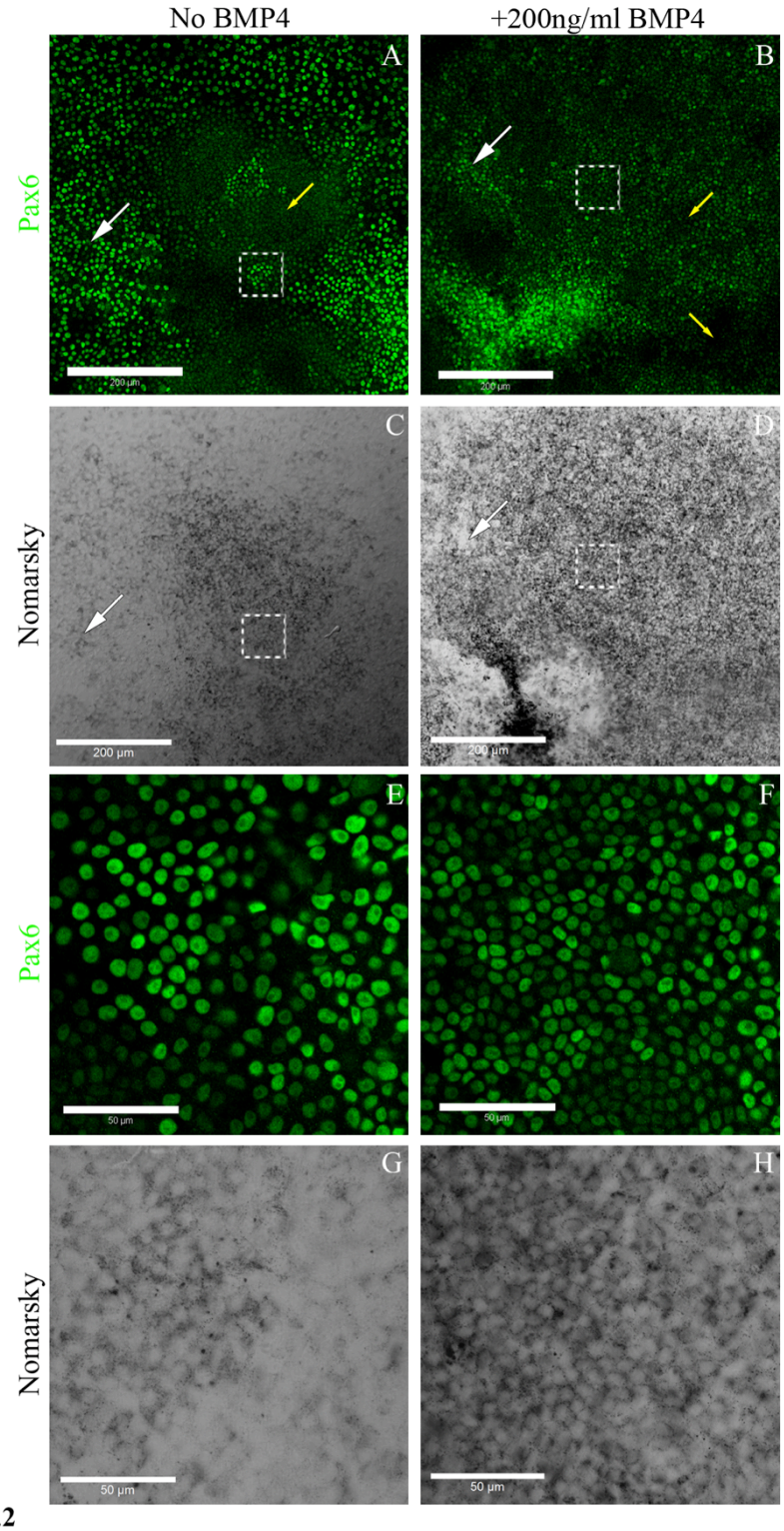


Fig. 4.2
Chick RPE HH21 explants cultured for 2 days +/-BMP4 (200ng/ml).
RPE explants were treated +/-BMP4 in order to ascertain whether or not BMP4 could down-regulate Pax6 expression after 2 days in culture. Pax6 expression in RPE cells was observed to be variable in both conditions with areas of both high and low expression. This unexpected change in Pax6 expression appeared to be independent of BMP4 treatement. -BMP4 (A, C, E, G), +BMP4 (B, D, F, H). Pax6 (green) (A, B, E, F), Nomarsky (C, D, G, H). E, G and F, H high mag. images of white box regions in A, C and B, D respectively. White arrows - high Pax6 expression, yellow arrows - low Pax6 expression. Scale bars: 200um (A-D), 50um (E-H).

be variable across a number of explants. Some areas of low Pax6 expression were located more centrally where one would expect to find quiescent RPE cells, as in Fig. 4.2A, however, other explants had these regions closer to the edge of the explant, as in Fig. 4.2B. Both centrally located, and peripherally located areas of low Pax6 expression were found in both BMP4-treated, and untreated explants, which would suggest that the down-regulation of Pax6 expression in the RPE cells was independent of the exogenous treatment with BMP4. What was clear is that these areas of low Pax6 expression were localized into regions of adjacent cells, rather than individual cells across the RPE monolayer, which may suggest that there is a region-specific reason for this observation; possibly resulting from localized, diffusible factors. The level of pigmentation was also observed to be variable across the RPE explants, however, once again this did not appear to be reliant on the treatment with exogenous BMP4, as explants in both conditions appeared to display a general pattern of lighter pigmentation being found at the edges, with darker pigmentation largely confined to the centre of the explants (Fig. 4.2C, D). However, the specific levels of pigmentation across the sheet appeared to be more variable within this general pattern, as evidenced in the higher magnification images (Fig. 4.2G, H). It was unclear simply through observation, whether or not the level of pigmentation was related to the level of expression of Pax6 in the explants. Some heavily pigmented areas displayed high expression of Pax6 (Fig. 4.2A-D white box, E-H) whereas others had less pigmentation but strong labeling for Pax6 (Fig. 4.2 A-D white arrows) and vice versa. To investigate any potential relationship between the pigmentation and the level of Pax6 expression, image analysis was employed in order to quantify the co-localized levels of Pax6 expression and pigmentation on low magnification images of the explants. There did not appear to be any strong relationship between the level of pigmentation and the expression level of Pax6 (Fig. 4.5A). The points represent 8 different regions of interest measurements for each of the BMP4-treated, and untreated, RPE explant images. They appear to be scattered in a largely random fashion with no apparent trend. Points measured from both conditions appear to cluster in a similar area of the graph and their overall averages are almost exactly the same (labeled +/-BMP whole of graph). The line of

best fit for explants treated with BMP4 (Fig. 4.5A blue line) appears to indicate that the pigmentation becomes lighter with increasing expression of Pax6, however, the R^2 value for this trend line (R^2=0.29) would suggest that this correlation is very weak, and therefore, the likelihood of there being a relationship between pigmentation levels of Pax6 expression is diminished. The untreated, negative control displayed a horizontal trend line, again with a very low R^2 value (R^2=0.00013), which would imply that there is no correlation between the pigmentation and Pax6 expression in this culture condition.

4.1.3.3 Effect of 2 day BMP5 treatment on Pax6 expression in explanted chick RPE HH21:

Again, Pax6 expression was maintained in both untreated, and BMP5-treated chick RPE explants after 2 days in culture (Fig. 4.3A, B, E, F). Both conditions exhibited the presence of RPE cells that were high in expression for Pax6 (Fig. 4.3E,F white arrows), with other areas expressing a lower amount of Pax6 (Figure 3A, B yellow arrows, E, F red arrows). These areas of low Pax6 expression once again appeared to be regional, rather than in individual cells. Several clusters of RPE cells expressing Pax6 at high levels could be observed as islands within the areas with less Pax6 expression (Fig. 4.3A, B white boxes, E, F white arrows). These were present in both BMP5-treated, and untreated conditions. The area of the regions of low Pax6 expression were observed to be highly variable in both culture conditions, with some explants exhibiting very large low Pax6 regions (as in Fig. 4.3A yellow arrow), with others exhibiting smaller regions (as in Fig. 4.3B yellow arrow). Once again, the level of pigmentation was found to be very variable, however, the most pigmented regions were generally localized to the centre of the RPE sheets, with lighter areas found at the leading edge of the explants (Fig. 4.3C, D). However, at higher magnifications, a more variable level of pigmentation was observed (Fig. 4.3G,H). The variable level in pigmentation did not appear to correlate with the level of expression of Pax6 (Fig. 4.3) as some areas of high Pax6 expression displayed only light pigmentation (for example Fig. 4.3E-H white arrows), as did low areas of Pax6 expression (for example Fig. 4.3E-H red arrows).

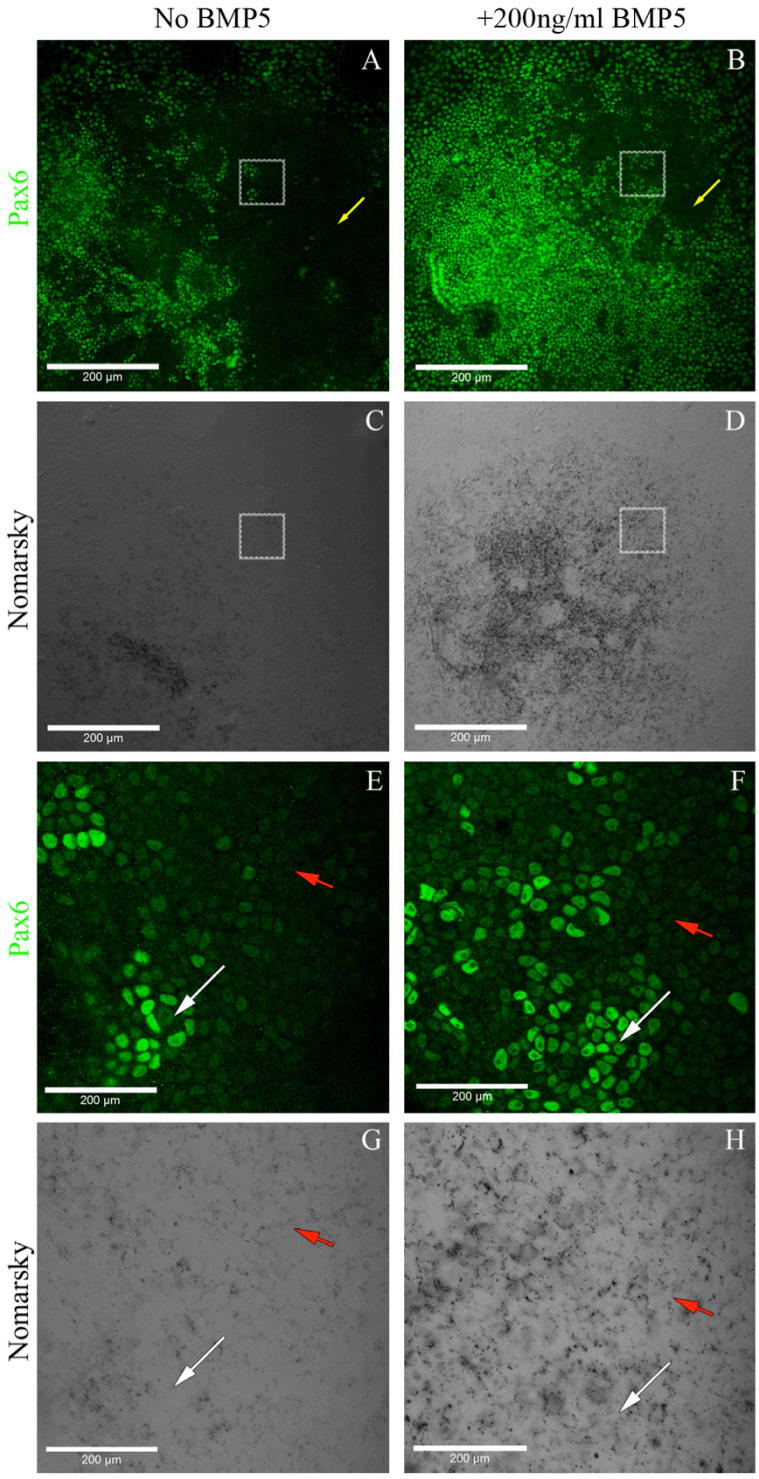


Fig. 4.3
Chick RPE HH21 explants cultured for 2 days +/-BMP5 (200ng/ml).
RPE explants were treated +/-BMP5 in order to ascertain whether or not BMP5 could down-regulate Pax6 expression after 2 days in culture. Pax6 expression in RPE cells was observed to be variable in both conditions with areas of both high and low expression. This unexpected change in Pax6 expression appeared to be independent of BMP5 treatement (A, B). -BMP5 (A, C, E, G), +BMP5 (B, D, F, H). Pax6 (green) (A, B, E, F), Nomarsky (C, D, G, H). E, G and F, H high mag. images of white box regions in A, C and B, D respectively. White arrows - Region of high Pax6 expression. Yellow/ red arrows - Region of low Pax6 expression. Scale bars: 200um (A-D), 50um (E-H).

In order to confirm this, image analysis was undertaken in order to quantify the relative levels of pigmentation in regions with differing expression of Pax6. The relative mean pixel intensity was measured for 8 different regions of interest in both BMP5-treated, and untreated explants. A scatterplot of these values would suggest that no correlation between the pigmentation of the RPE cells and the level of expression of Pax6 exists, owing to the fact that data points for both conditions over-lap on the graph, and appear to have a random organization (Fig. 4.5B). The line of best fit for both of the conditions appears to show a negative correlation between pigmentation and the level of Pax6, which would suggest that Pax6 expression decreases as cells become lighter in pigmentation. However, in both BMP5-treated (blue data points/trend line), and untreated controls (red data points/line), the R^2 value is very low (R^2=0.12, 0.035 respectively) which implies that the likelihood of the existence of a relationship between Pax6 expression and pigmentation is remote.

4.1.3.4 Effect of 2 day BMP7 treatment on Pax6 expression in explanted chick RPE HH21:

Once again, Pax6 expression was present in both BMP7-treated, and untreated RPE explant cultures after 2 days in culture (Fig. Fig. 4.4). This expression was varied throughout the RPE sheets, again with areas of low Pax6 expression (Fig. 4.4A, B yellow arrows, E, F red arrows) contrasting with areas of high expression (Fig. 4.4A, B, E, F white arrows). The size of these areas was variable as observed previously in cultures treated with other BMP family signaling molecules. Areas of the RPE sheet with low Pax6 expression tended to exist in clusters, which may suggest a localized mechanism for down-regulation of Pax6. There did not appear to be a difference in the overall level of Pax6 expression between explants treated with BMP7, and those which were untreated (Fig. 4.4A, B, E, F). This would suggest that the effect is not dependent upon exogenously added factors, but instead is an intrinsic property of either the RPE itself, or an artifact of the culture system. In addition, the pigmentation of the RPE did not appear to be affected by the treatment with BMP4 as RPE in both conditions displayed very variable levels of pigmentation throughout the sheet (Fig. 4.4C,D, G, H). For

example, Fig. 4.4D displays an area at the centre of the explant, which is very densely pigmented. Conversely, other areas of the sheet appear much less pigmented. Similarly, there was no obvious correlation between the level of pigmentation, and the expression of Pax6, as some areas with both high and low expression of Pax6 appeared to exhibit similar levels of pigmentation (Fig. 4.4E-H white arrows high expression, red arrows low expression of Pax6). Despite this, it was difficult to ascertain whether or not there is a relationship between pigmentation and Pax6 by eye. Therefore, it was necessary to again employ the use of image analysis software to sample a number of different regions with variable Pax6 and pigmentation levels, in order to measure their corresponding levels in these regions. A scatterplot of 8 different sampling regions suggests that there is little, if any difference between the overall Pax6/pigmentation of the entire image, given that the average points are plotted very close together (Fig. 4.5C labeled +/-BMP7 Whole). As one would expect therefore, the data points from each of the 8 samples in both +/-BMP7 conditions appear to cluster in the same areas of the graph. The line of best fit for +BMP7 data points suggests that there is a possible negative correlation between the level of Pax6 and the level of pigmentation (Fig. 4.5C blue line). This would suggest that Pax6 expression decreases with lighter pigmentation, however, given that the R^2 value for this trend line is very low (R^2=0.06), this would suggest that only a very, very weak correlation exists. This would imply that the likelihood of there being a relationship between the levels of pigmentation is remote. Similarly, the line of best fit for the untreated condition suggests a similar negative correlation between Pax6 expression and pigmentation, albeit a less severe correlation as demonstrated by a smaller gradient. However, in this case, the R^2 value is much higher (R^2=0.7), which suggests a relatively strong relationship between the level of Pax6 expression and pigmentation. The fact that no similar correlation has been observed in negative controls for BMP4 or BMP5 assays casts doubt on whether or not this result is real, or is in fact an artifact of the small dataset.

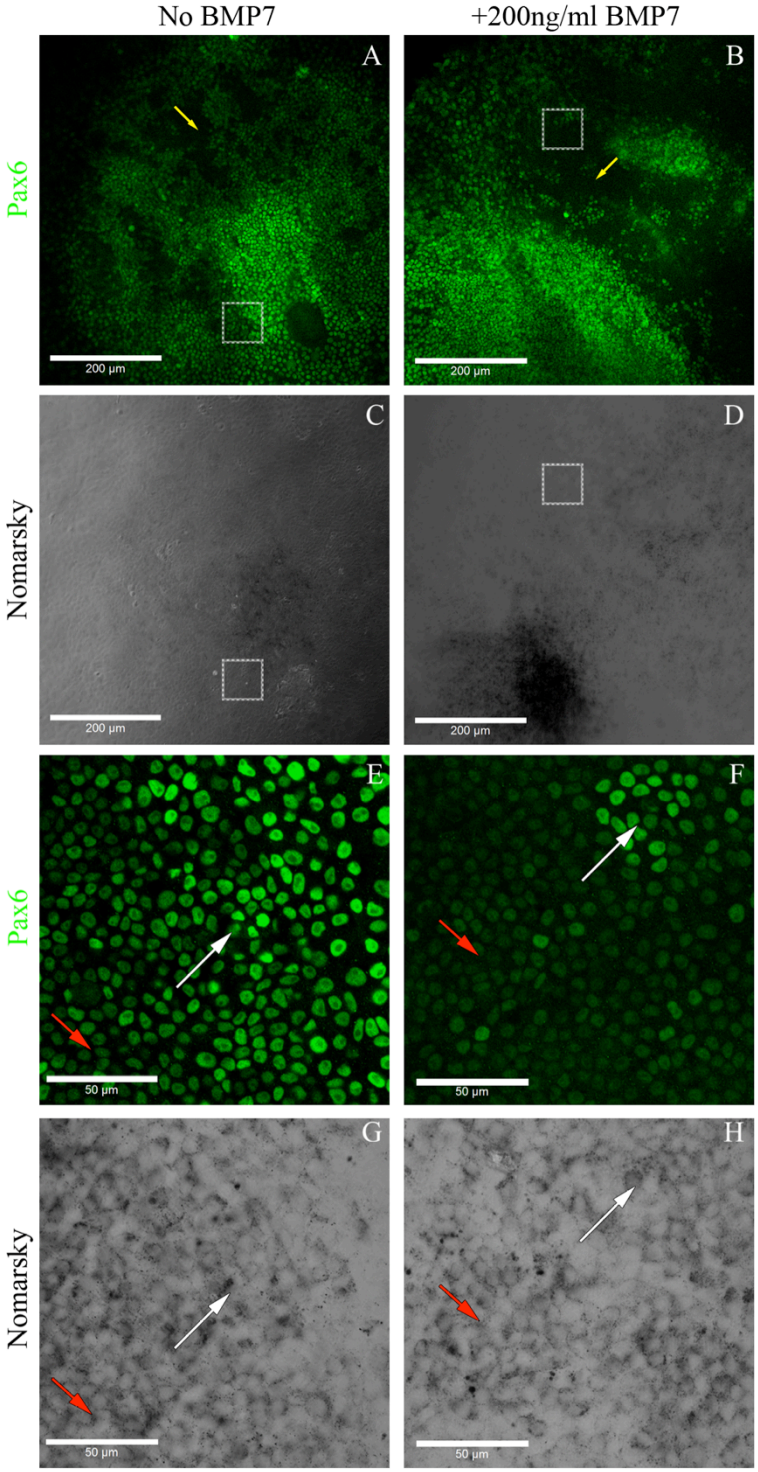


Fig. 4.4

Chick RPE HH21 explants cultured for 2 days +/-BMP7 (200ng/ml).

RPE explants were treated +/-BMP7 in order to ascertain whether or not BMP7 could down-regulate Pax6 expression after 2 days in culture. Pax6 expression in RPE cells was observed to be variable in both conditions with areas of both high and low expression. This unexpected change in Pax6 expression appeared to be independent of BMP7 treatement given that this variation in the levels of Pax6 was observed in both culture conditions. -BMP7 (A, C, E, G), +BMP7 (B, D, F, H). Pax6 (green) (A, B, E, F), Nomarsky (C, D, G, H). E, G and F, H high mag. images of white box regions in A, C and B, D respectively. White arrows - Regions of high Pax6 expression. Yellow/ red arrows - Regions of low Pax6 expression. Scale bars: 200um (A-D), 50um (E-H).

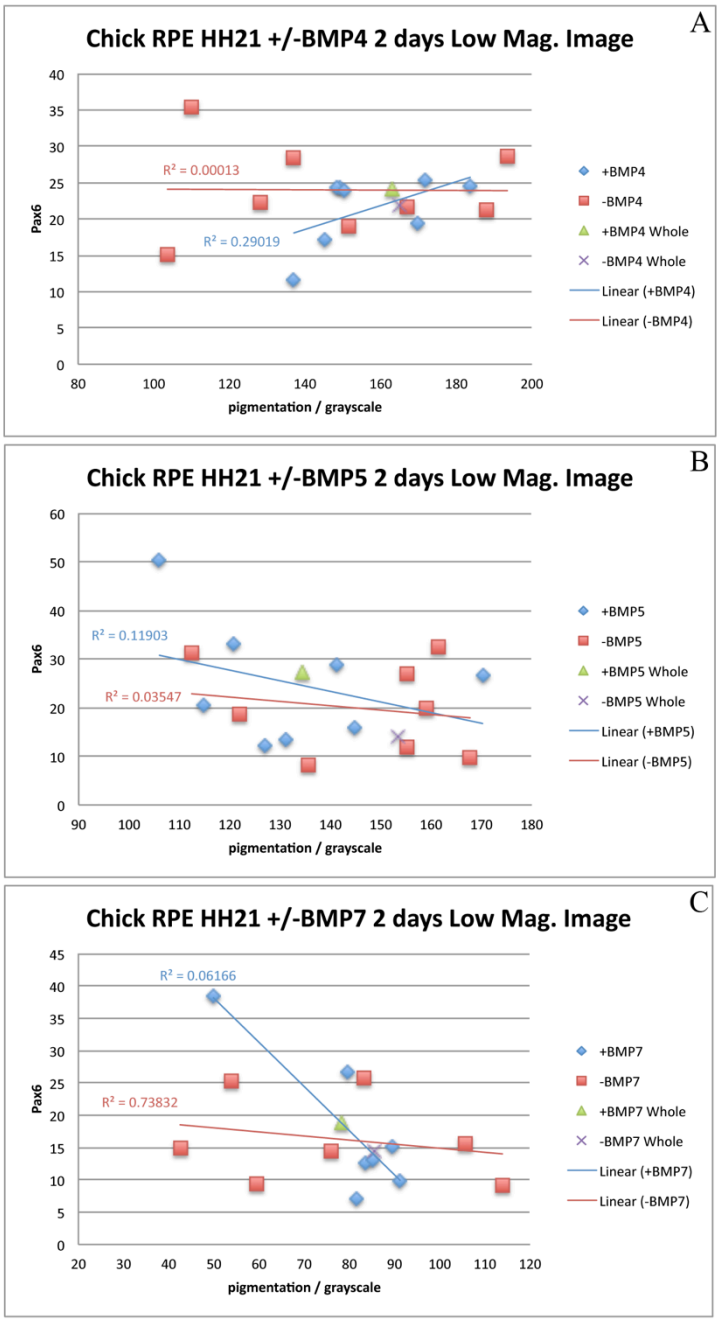


Fig. 4.5
Relative levels of Pax6 expression vs pigmentation in +/- BMP4 (A), +/- BMP5 (B), and +/- BMP7 (C) (200ng/ml) treated Chick RPE HH21 explants cultured for 2 days. In order to ascertain whether area of varying Pax6 expression correlated with the level of pigmentation in RPE cells cultured for 2 days, image analysis software was employed to quantify the relative Pax6 expression and pigmentation in 8, equally-sized, regions of interest. Pax6 expression for each image was measured by quantifying the intensity of fluorescence for each pixel, within each region of interest, after converting images to grayscale (0 = Black, 255 = White). Mean fluorescence intensity for each region was subsequently expressed as the mean pixel intensity of each region, measured on a grayscale. No strong correlation between the level of pigmentation and the expression level of Pax6, in the corresponding region of each image, which is displayed by the scattered appearance of these graphs, in addition to the low R squared values.

4.1.4 Discussion:

The data from these experiments would suggest that BMP signaling factor members, BMP4, BMP5, and BMP7 are not able to directly modulate the expression of Pax6, or the level of pigmentation in cultured RPE explants at stage HH21.

Treatment of HH21 RPE explants with a high concentration of BMP4 for 1 day did not yield any change in the expression of Pax6 within the RPE cells, and all cells of the explants were observed to express the transcription factor. Given the evidence that BMP family members are strongly implicated in the augmentation of the RPE phenotype during development, and therefore the potential down-regulation of Pax6 in the RPE as cells mature (Muller et al., 2007, Zhang and Yang, 2001), this was a surprising result. It was therefore assumed that perhaps 1 day was not long enough in culture for the cells to respond to the exogenous growth factor treatment, and therefore, the assay was repeated with a 2-day culture period. Additionally, despite the reported RPE-specification effects of BMP4 in the developing optic cup, including induction of RPE markers like MMP115 in retinal neuroepithelium following application into the eyecup (Muller et al., 2007), the evidence that BMP signaling may regulate the expression of Pax6 was indirect. Application of a soluble BMP-antagonist, noggin, into the developing optic cup was shown to result in an up-regulation of Pax6 in RPE, and a subsequent induction of RPE to neural retina transdifferentiation. Noggin acts to inhibit BMP-family growth factors by binding them in solution, thereby preventing the interaction of BMPs with their cell-surface receptors. However, noggin is known to bind a number of different BMP signaling family members, and its affinity for each one is varied (Belecky-Adams and Adler, 2001). It is possible therefore that inhibition of another member of the BMP signaling family is responsible for the observed effect on Pax6 expression, and subsequently transdifferentiation. BMP5 and BMP7 are both reported to be expressed more strongly in the RPE than BMP4, which is largely restricted to the dorsal retina during development (Belecky-Adams and Adler, 2001). Therefore, it is perhaps reasonable to assume that BMP5 and BMP7 are more likely to undergo autocrine

signaling in the RPE than BMP4, which may contribute to the limitation in potential for transdifferentiation with development. BMP signaling passes through intra-cellular, down-stream effector proteins known as Smad proteins (Belecky-Adams and Adler, 2001). These proteins act as cytosolic receptors that are able to transduce the BMP signal, via the recruitment of additional factors, to influence transcriptional output. BMP signaling employs the action of specific members of the Smad family, Smads 1, 5 and 8. These receptor Smads are activated in different combinations depending upon which BMP signal is received. This in turn regulates the different changes in gene expression. Therefore, given the fact that different BMP signaling proteins have different effectors, a selection of BMPs with different targets were tested for their effect on the expression of Pax6, in cultured RPE explants.

Once again, no consistent difference in the pigmentation, or expression level of Pax6, was observed between any BMP4, BMP5 and BMP7 treated explants, when compared with their negative controls. However, the RPE explants did show variability in the level of Pax6 expression across the explanted sheets in both treated, and untreated culture conditions. This would again suggest that BMP signaling is not able to act directly act on cultured RPE cells and regulate Pax6 expression, or indeed their pigmentation. Previous experiments where BMP-antagonists were shown to effect the expression of Pax6 were applied at a very early stage of development (Muller et al., 2007), prior to the invagination of the optic cup (HH8/9). Therefore, it is possible that BMP signaling can directly regulate the expression of Pax6 at early stages, but does not regulate the expression of Pax6 in RPE cells at this later stage (HH21). However, this seems unlikely given that other factors involved in the specification of both layers of the multi-potent optic cup, both RPE and neural retina, persist in an antagonistic gradient throughout the development of the optic cup, for example, activin (Fuhrmann et al., 2000b) and FGFs (Vogel-Hopker et al., 2000, Pittack et al., 1997, Nguyen and Arnheiter, 2000) respectively. These factors are not only functional at specific stages, as changes in the balance between RPE-fate augmentation, and retinal-fate augmentation, at any stage prior to HH24, results in the RPE gaining a retinal phenotype (Pittack et al., 1997, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993), and the RPE

gaining an RPE like phenotype (Muller et al., 2007, Fuhrmann et al., 2000b). Additionally, ectopic BMP application at later stages in the optic cup (approximately HH17) was still able to induce RPE-specific gene expression within the neuroepithelium of the developing retina, which suggests that the action of BMP family members in the specification of RPE cell fate is still active in the optic cup at later stages.

It was not possible to confirm whether BMP growth factors would modulate the expression of Pax6 in RPE explants taken from a comparable stage as previous experiments (HH8/9). At these stages, the eye is very small and the RPE is not yet pigmented. Both of these factors combine to make it near impossible to obtain intact RPE explants, which are free of other contaminant tissues.

The RPE fate augmentation effects of BMP growth factors may act through a Pax6-independent pathway, which is why no change in the level of expression of Pax6 was observed. This again seems unlikely given the fact that there was also no consistent increase in the level of pigmentation, as could reasonably be expected of any treatment which augments the RPE phenotype. Additionally, the reported effects of noggin-application, and therefore, BMP inhibition, in increasing the expression of Pax6 would suggest that the RPE specification effects of BMP signaling do involve a Pax6-dependent element. Taken together, these data suggest that BMP signaling factors may not act directly upon RPE cells in culture, but may actually employ secondary pathways, which require the presence of other tissues in order to modulate the expression of Pax6.

The variation in the levels of expression of Pax6 across the explanted RPE sheets was unexpected, with some areas maintaining robust Pax6 expression, whereas others had barely detectable expression of the transcription factor. The apparent down-regulation of Pax6 expression in some areas of the sheet appeared to occur independently of the treatment with exogenous growth factors, which would suggest that this down-regulation was an artifact of the culture system, or it may imply that the RPE have an intrinsic ability to regulate its own expression of Pax6, without the need for other tissues. It is possible that the extracellular matrix on which the RPE explants were plated may have some effect on the expression of

Pax6 in the RPE cells, however, this seems unlikely given that other RPE cell cultures which use Matrigel as a coating are able to maintain their expression of the transcription factor for a long period of time. For example, HESC-RPE cultures can maintain Pax6 expression for at least 5 weeks when cultured on Matrigel (Vugler et al., 2008). It is possible that some component of the culture medium, for example a component of the fetal bovine serum, could be responsible for this down-regulation. However, this may be unlikely given that chick RPE is able to undergo transdifferentiation, which requires an up-regulation of Pax6 (Spence et al., 2007b, Azuma et al., 2005a), in the same culture medium (see Chapter 3). The high concentration of bFGF employed to induce transdifferentiation in these experiments may be able to overcome any inhibitory factor that may be present in the serum, and therefore, this still remains a possibility. However, the localized clustering of regions with low Pax6 expression might not be expected to be found if the factor responsible is uniformly present throughout the culture system, as would be the case if a medium component, or extracellular matrix were to be responsible.

RPE cells generally become more pigmented as the developmental stage increases *in vivo*, and therefore, it was suspected that this might provide evidence that RPE cells low in Pax6 expression would be more pigmented, thereby indicating that they are later in their development. However, quantitative image analysis showed that there was no strong correlation between the level of expression of Pax6 and the corresponding level of pigmentation, as evidenced by the very low R^2 values for the trend lines associated with these datapoints. Only one dataset showed evidence of a strong correlation between the two variables, the points for untreated explants in the BMP7 assay. These datapoints displayed a slight negative correlation, with an R^2 value of approximately 0.7, which suggests that Pax6 is reduced as cells lose their pigmentation. However, this result appears unreliable given that other untreated explants used as controls in the other assays, which were experimentally equivalent to this result, did not show any strong correlation. It may be that this result is an artifact of this particular image, or may reflect that the low number of sampling points in insufficient to draw concrete conclusions. Untreated RPE explants cultured for a number of

days did not show any particular increase in the level of pigmentation, which may in fact suggest that pigmentation is not an effective measure of developmental stage *in vitro*.

Despite the fact that no exogenous growth factor-dependent changes in Pax6 expression appeared to occur in these assays, the unexpected, growth factor independent, down-regulation of some areas of Pax6 in the explants after 2 days made it difficult to accurately determine whether or not BMP growth factors could effect the expression of Pax6 in cultured monolayers. It was therefore necessary to employ the use of another assay, similar to that employed by Sakami et al (Sakami et al., 2008), in order to ascertain the whether BMP growth factors have any inhibitory effects on the onset of bFGF-induced RPE transdifferentiation.

4.2 Can exogenously added BMP signaling family members inhibit the bFGF-mediated transdifferentiation of chick RPE HH24?

4.2.1 Introduction:

The previous assay did not appear to show any effect of BMP4, BMP5 or BMP7 on the expression of Pax6, a master transcriptional regulator of transdifferentiation (Spence et al., 2007b, Azuma et al., 2005a, Kuriyama et al., 2009a). However, the fact that the RPE explants appear to have an intrinsic ability to down-regulate the expression of Pax6 independently of the treatment with exogenous factors, and that this effect is highly variable, makes it difficult to quantify any changes in the expression of Pax6 that may occur as a result of BMP-treatment. It was therefore necessary to investigate the effect of BMP growth factors on the regulation of transdifferentiation more directly. If BMP signaling factors are able to restrict the ability of chick RPE cells to undergo transdifferentiation in response to bFGF, then exogenous addition of BMP factors to bFGF-treated, RPE explants should be able to inhibit the onset of transdifferentiation.

4.2.2 Materials & Methods:

4.2.2.1 RPE isolation & culture:

Embryonic chick RPE HH24 was cultured in the standard, non-adherent, transdifferentiation culture system for 7 days as described in chapter 2. Explants were cultured in DMEM/F12 + 1% FBS for reasons discussed previously. Each explant was divided into 3 approximately equally sized sheets for 3 different culture conditions: +bFGF (100ng/ml), +bFGF (100ng/ml)(positive control), + BMP4/5/7 (200ng/ml)(experimental condition), untreated (negative control) (See figure 4.4M for schematic demonstration of culture protocol).

4.2.2.2 Immunohistochemistry, statistics & image analysis:

Immunohistochemistry and statistics were performed as described in chapter 2.4. Image analysis was performed as described in the previous experiment, with thresholding being employed for fluorescent marker quantification, and the *region of interest* tool being used the quantify pigmentation (Fig. 4.5M). Statistical analysis was performed as in chapter 2.11.

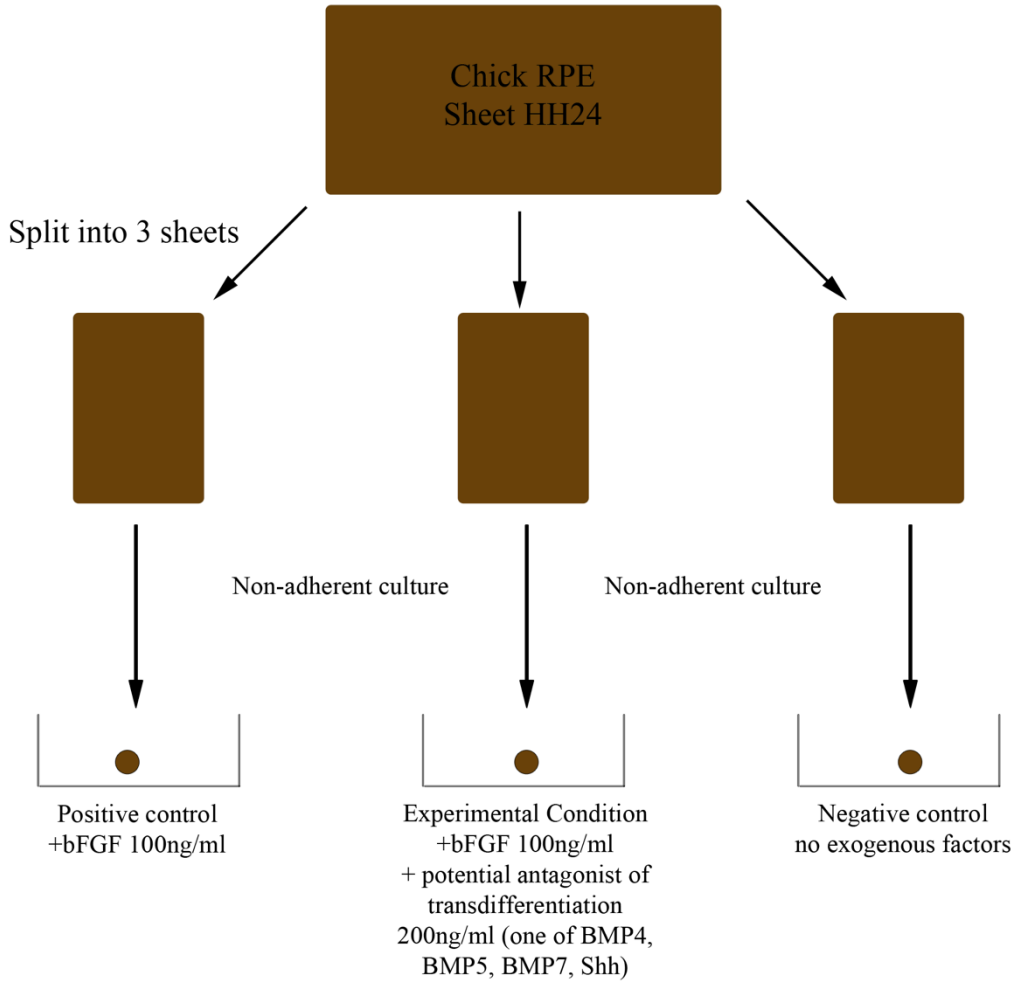


Fig. 4.4-M
Preparation and culture of RPE HH24 explants in order to assay the potential of BMP/Shh growth factors to inhibit bFGF-mediated transdifferentiation.
A single embryonic chick RPE sheet HH24 was dissected from the central region at the back of the eye, ensuring all adjacent tissues were removed. This single sheet was then divided into 3 approximately equally sized sheets and treated with one of 3 different culture conditions as described above.

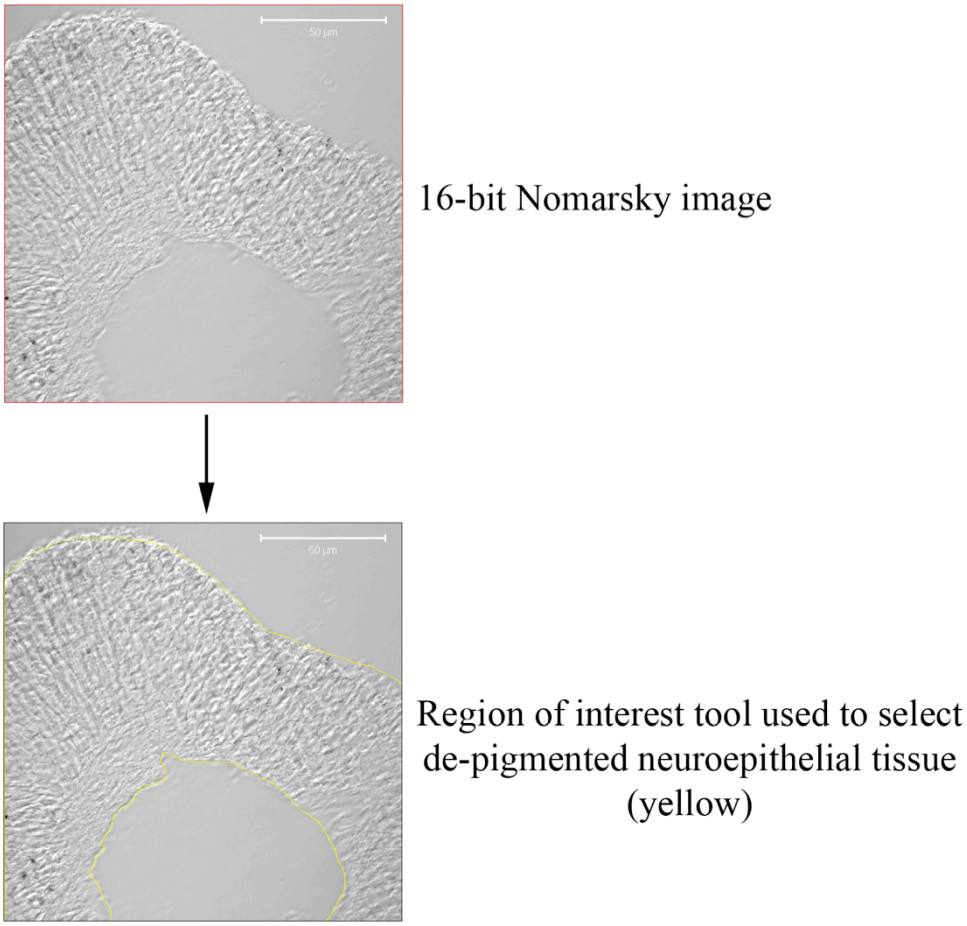


Fig. 4.5-M
Method of region of interest selection in order to quantify changes in the level of pigmentation in treated RPE explants HH24 in comparison to untreated explants.
De-pigmented, transdifferentiated neuroepithelium was selected using a 'region of interest' tool so as to include all areas (yellow line), both de-pigmented areas in addition to pigmented patches.

4.2.3 Results:

4.2.3.1 BMP4:

Culture of chick RPE stage HH24 explants in the presence of bFGF and BMP4 did not result in an inhibition of the onset of transdifferentiation. RPE explants were observed to lose their characteristic RPE morphology and transdifferentiate into tissue displaying retinal phenotype (Fig. 4.6i; 4.6ii, 4.7). This included the expression of retinal markers of transdifferentiation, including Pax6 (Fig. 4.6iD, F), Sox2 (Fig. 4.6iD, E), HuD (Fig. 4.6iM, O). No rhodopsin expression was observed in this explant (Fig. 4.6iM, N), however, as previously discussed, the expression of rhodopsin is very variable and has not been found in all transdifferentiated explants. The analysis of the expression of rhodopsin is used in this assay as an extra indicator of a retinal fate, which further supports any transdifferentiation that may have taken place. Immunohistochemical analysis of these retinal markers appears to suggest that their expression levels may be slightly reduced when cultured in the presence of bFGF+BMP4, in comparison to the embryo-matched, +bFGF, positive control condition. Image analysis of the expression levels of Pax6, Sox2 and HuD in each condition are displayed in Fig. 4.6ii which shows that the average level of expression of each marker is indeed reduced in bFGF+BMP4 treated explants, when compared to the positive control condition, however, this difference was not found to be significant (n=3). The relatively large size of the standard error bars on the graph indicates the large amount of variability in the assay. Similarly, the expression of retinal markers was largely absent from the untreated, negative control explants, however, some Pax6 expression was observed in a few of these explants but neither Sox2 or HuD expression was ever present (n=3). The negative control condition was shown to be significantly different from the other two conditions, which indicates the differences between transdifferentiated RPE cells which subsequently acquire a retinal phenotype, and untransdifferentiated RPE explants which do not express retinal markers. Despite this, the pattern of expression for each of the retinal markers was not found

to be statistically significant (RANOVA n=3). It is possible that the large amount of variability and the small sample size are responsible for this.

In addition to the expression of retinal markers associated with RPE to neural retina transdifferentiation, RPE explants treated with both bFGF and bFGF+BMP4 were also observed to lose their characteristic pigmentation in association with transdifferentiation (Fig. 4.7A, B respectively), when compared to untreated controls (Fig. 4.7C). Pigmentation in both conditions where bFGF was present was greatly reduced, primarily at the surface of the aggregated explants (Fig. 4.7A, B). These areas appeared to take on a pseudo-laminar, retinal-like structure characteristic of transdifferentiated RPE. These largely de-pigmented areas did still contain some pigment, however this was greatly reduced when compared the negative controls. The majority of the pigment that still resided in transdifferentiated RPE explants was in the centre of the aggregates, as previously discussed in chapter 3. Image analysis showed that explants treated with bFGF+BMP4 had slightly more average levels of pigmentation than +bFGF, positive controls, however, the standard error bars for these two treatments over-lapped which indicates that the difference is not significant (Fig. 4.7D). This was consistent with the apparent decrease in fluorescence of retinal markers between +bFGF and +bFGF+BMP4 treated explants (Fig. 4.6i; 4.6ii), which could suggest that addition of BMP4 to the culture is able to slightly inhibit the retinal phenotype. However, this difference was not significant. +bFGF and +bFGF+BMP4 treated explants were found to contain significantly lower levels of pigmentation that untreated controls (Fig. 4.7D) as expected for RPE cells which retained their characteristic, pigmented phenotype, and did not transdifferentiate. The overall pattern of pigmentation levels between the three culture conditions was found to be statistically significant (Fig. 4.7D, RANOVA, $p<0.05$ n=3).

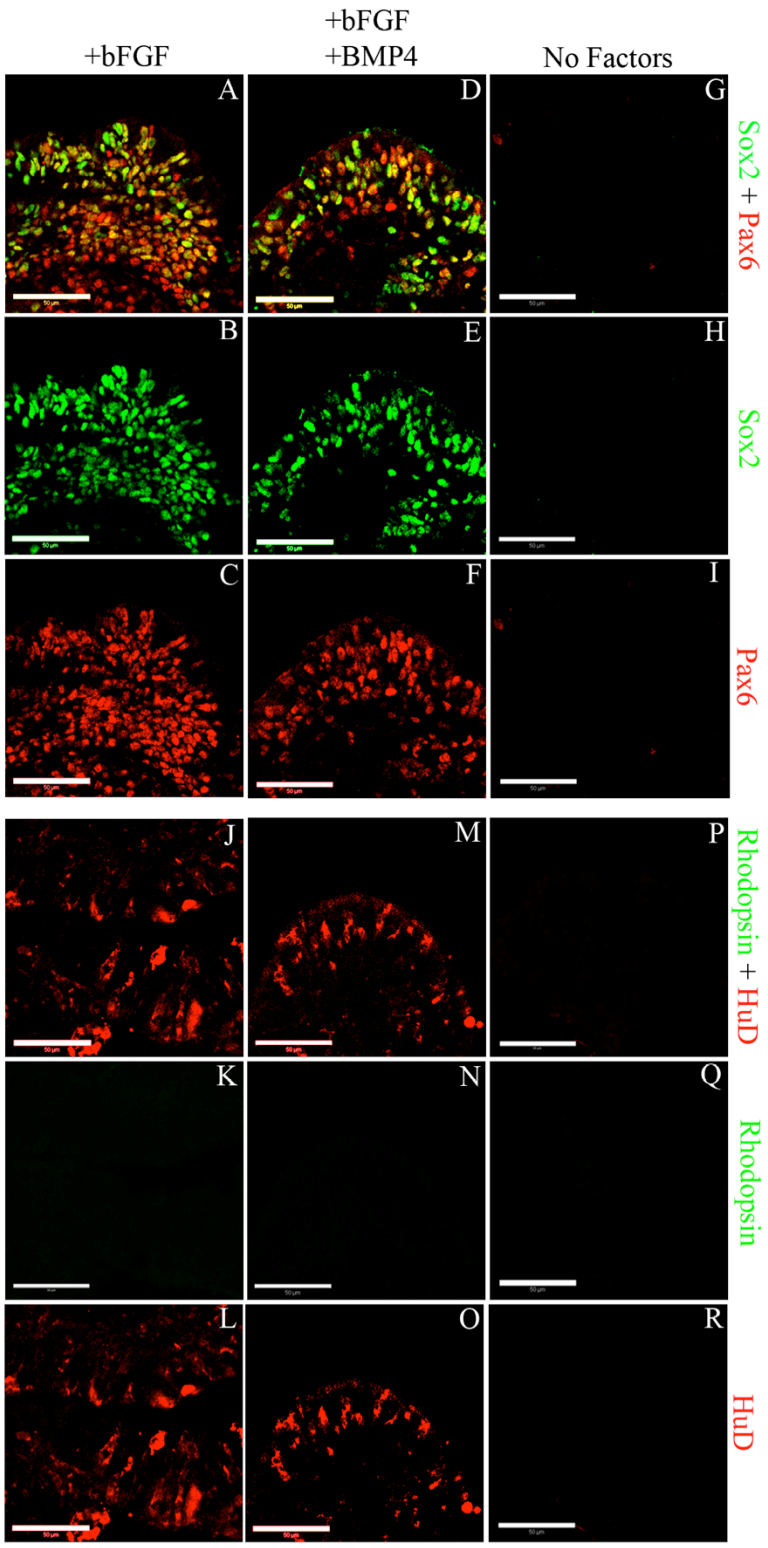


Fig. 4.6i

BMP4 (200ng/ml) does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture.

RPE treated with +bFGF (A-C, J-L) and +bFGF+BMP4 (D-F, M-O) both displayed similar characteristics of transdifferentiation through expression of retinal markers, Pax6 (A, C and D, F respectively), Sox2 (A, B and D, E respectively) and HuD (J, L and M, O respectively) in a neuroepithelial pattern. Untreated controls did not express retinal markers is most instances (G-R), except some untreated explants did retain the expression of Pax6. (n=3). Error bars: standard error. Scale bars: 50um.

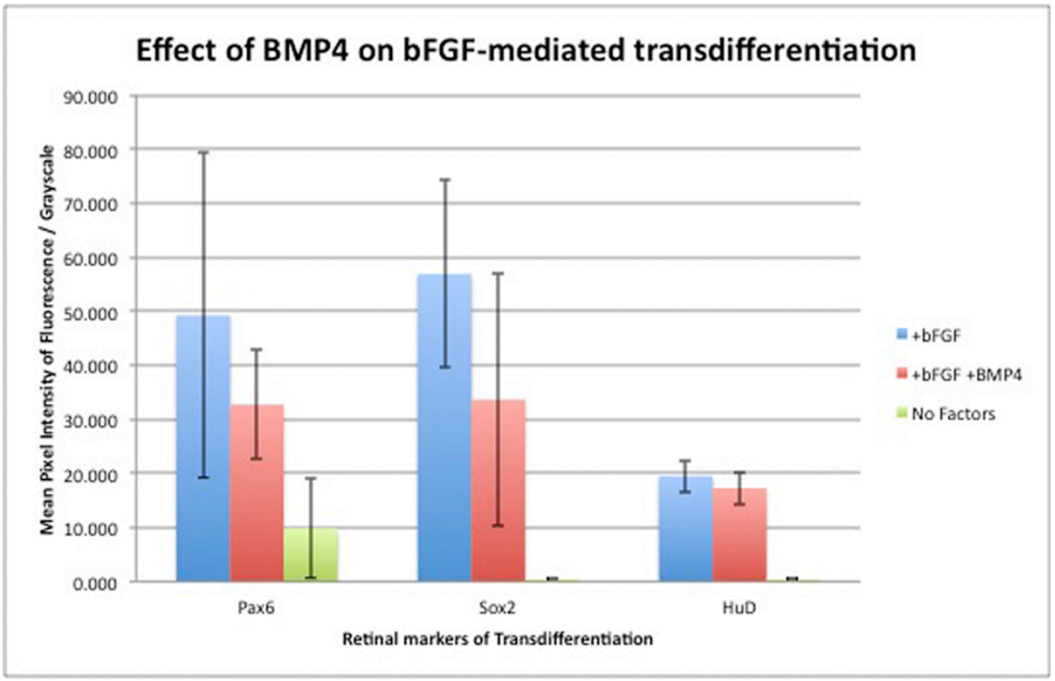


Fig. 4.6ii

Fluoresence quantification of retinal marker expression in chick RPE transdifferentiation assay to observe the effect of BMP4 (200ng/ml) of bFGF (100ng/ml) mediated transdifferentiation.

BMP4 does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture as the level of expression of Pax6, Sox2, and HuD in +bFGF (Blue bars) and +bFGF/+BMP4 (Red bars) cultures were not significantly different in their level of expression. Retinal marker expression was largely very low, or absent from no growth factor controls (Green bars). n = 3. Error bars: Standard error.

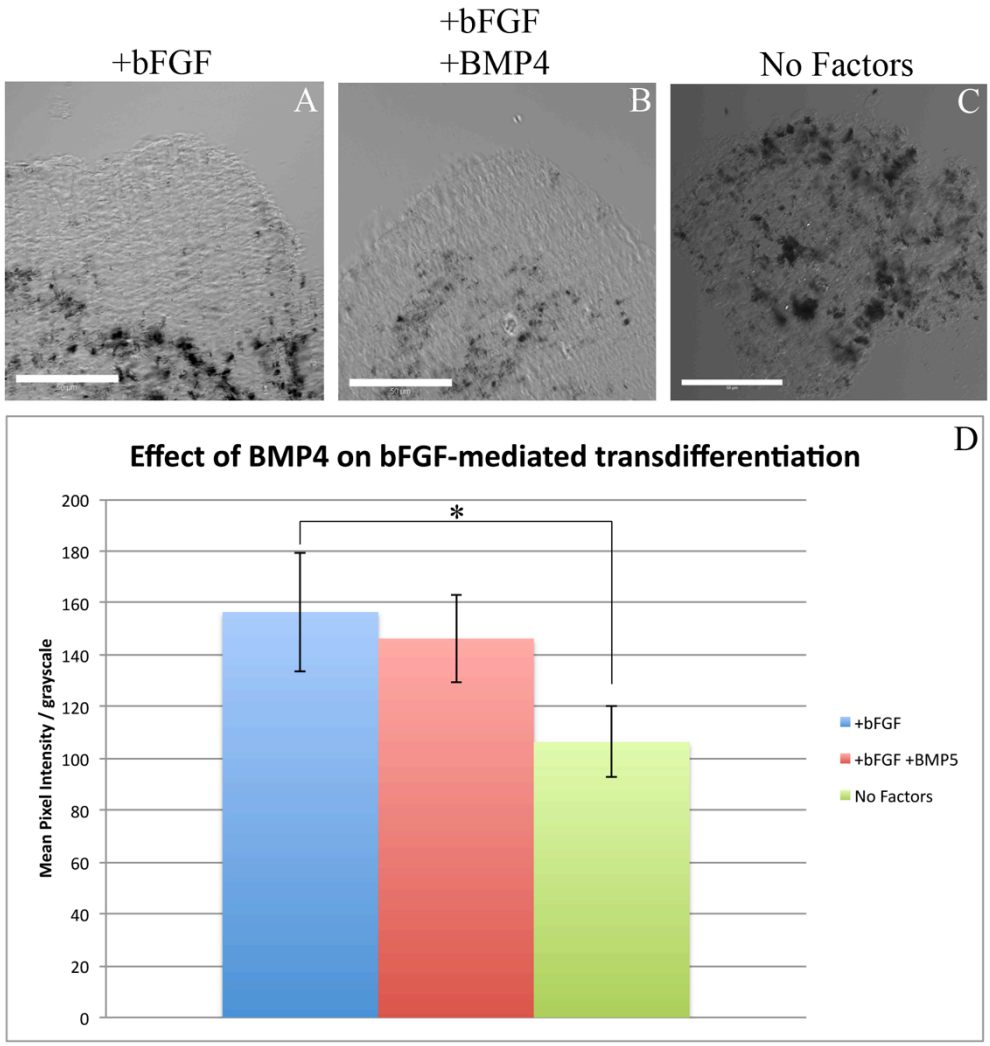


Fig. 4.7

BMP4 (200ng/ml) does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture.

RPE explants treated with +bFGF (A) and +bFGF+BMP4 (B) both displayed significant decrease in the level of pigmentation when compared with untreated controls (C). This is a classical characteristic of RPE to neural retina transdifferentiation which indicates that BMP4 does not inhibit bFGF-mediated onset of transdifferentiation. The graph shows the relative levels of pigmentation of explants in each culture condition after 7 days. This was quantified using image analysis software which gave the average pixel intensity of each pixel, in a selected area of the image. This selected area encompassed the whole tissue region only for each image analysed. No background was included. Untreated explants are significantly more pigmentated than growth factor treated explants. Error bars: standard error. Scale bars: 50um (* $p<0.05$, RANOVA, (n=3)).

4.2.3.2 BMP7:

The same assay using BMP7 growth factor in the place of BMP4 growth factor yielded similar results. RPE explants treated for 7 days with either +bFGF or +bFGF+BMP7 both displayed evidence of RPE to neural retina transdifferentiation (Fig. 4.8i; 4.8ii), which suggests that BMP7 does not restrict the capacity for transdifferentiation in response to bFGF. Explants treated with either bFGF, or bFGF+BMP7 were both positive for retinal markers, Pax6 (Fig. 4.8iA, C, G, I), Sox2 (Fig. 4.8iA, B, G, H), HuD (Fig. 4.8iD, F, J, L) and Rhodopsin (Fig. 4.8i D, E, J, K), whereas only Pax6 was detected in the untreated, negative controls (Fig. 4.8iM-R). As previously discussed, Pax6 was absent from the majority of negative controls, however, in this instance, the transcription factor continued to be expressed at a low level after 7 days in culture in some explants (Fig. 4.8iM, O). The Pax6 and Sox2 expression in +bFGF and +bFGF+BMP7 conditions closely resembled the expression of these key transcription factors during retinal development. These markers were mostly expressed throughout a neuroepithelial structure, which had resulted from transdifferentiation of the RPE. The intensity of the expression of both Pax6 and Sox2 appeared to be consistent between the two culture conditions. There was a possibility that the majority of expression of both Pax6 and Sox2 was confined to the basal side of the neuroepithelium, which in transdifferentiated RPE corresponds to the developing INL/ganglion cells layers of the retina. This inverted phenotype supports the idea that the observed retinal tissue has resulted from transdifferentiation of the RPE, and not another source, such as retinal contamination. Many, if not all of the Sox2 positive cells were observed to co-localise with Pax6 positive cells, as would be expected for developing retinal progenitors, and is consistent with the evidence that they interact with one another during transcriptional regulation (Aota et al., 2003, Ma et al., 2009, Oron-Karni et al., 2008, Matsushima et al., 2011, Lin et al., 2009). However, not all of the Pax6 positive cells in these transdifferentiated retinas co-localised with Sox2 (Fig. 4.8iA, G) expression. The most intensely labeled Pax6 nuclei were largely absent for Sox2 expression and were largely confined to the basal layer of the neuroepithelium (Fig. 4.8iC, I),

which is where the ganglion cells are born (Nishina et al., 1999, Prada et al., 1991, Prada et al., 1992). Cells expressing similarly high levels of Pax6, but located in the middle of the neuroepithelium, could also be developing ganglion cells. Ganglion cells are known to migrate from the middle of the neuroepithelium, towards the basal surface of the transdifferentiated neuroepithelium (Bovolenta et al., 1997). The Pax6 expression present in some of the untreated controls appeared to be lower in intensity that that found in the transdifferentiated retinas (Fig. 4.8iM, O), which would be consistent with the idea that a bFGF-mediated, up-regulation in Pax6 is required for transdifferentiation and development of a novel retina (Spence et al., 2007b). This expression did not appear to have any organized pattern as in transdifferentiated retinas, and was spread throughout the untreated explant (Fig. 4.8iM, O). The expression of ganglion and amacrine cell marker, HuD was largely confined to the basal layer of the transdifferentiated neuroepithelia resulting from both +bFGF (Fig. 4.8iD, F), and +bFGF+BMP7 (Fig. 4.8J, L) treatment, once again indicating the inverse nature of the transdifferentiated retina. However, cells expressing the protein were found throughout the neuroepithelium in both conditions, possibly as a result of the disorganized conformation of some transdifferentiated explants, which would surely effect the spatio-temporal development of the novel retinae, and subsequently the expression patterns of retinal markers. Despite this, RPE explants cultured in both of these conditions were observed to express rhodopsin in a handful of cells located at the apical surface of the neuroepithlium. This is where one would normally expect to find the developing photoreceptor layer in an inverted, transdifferentiated retina (Opas and Dziak, 1994a). Rhodopsin was observed in the cell membrane of these cells, which is where the protein would normally reside. The number of rhodopsin positive cells was very low in explants of both conditions, with some explants not expressing the protein at all. Therefore, the data is perhaps insufficient to analyse whether or not there is a difference in the level of intensity of expression/number of positive cells, between the positive +bFGF treatment, and the experimental +bFGF+BMP7 conditions. Untreated RPE explants did not display expression of either HuD or Rhodopsin (Fig. 4.8iP-R). Image analysis of average

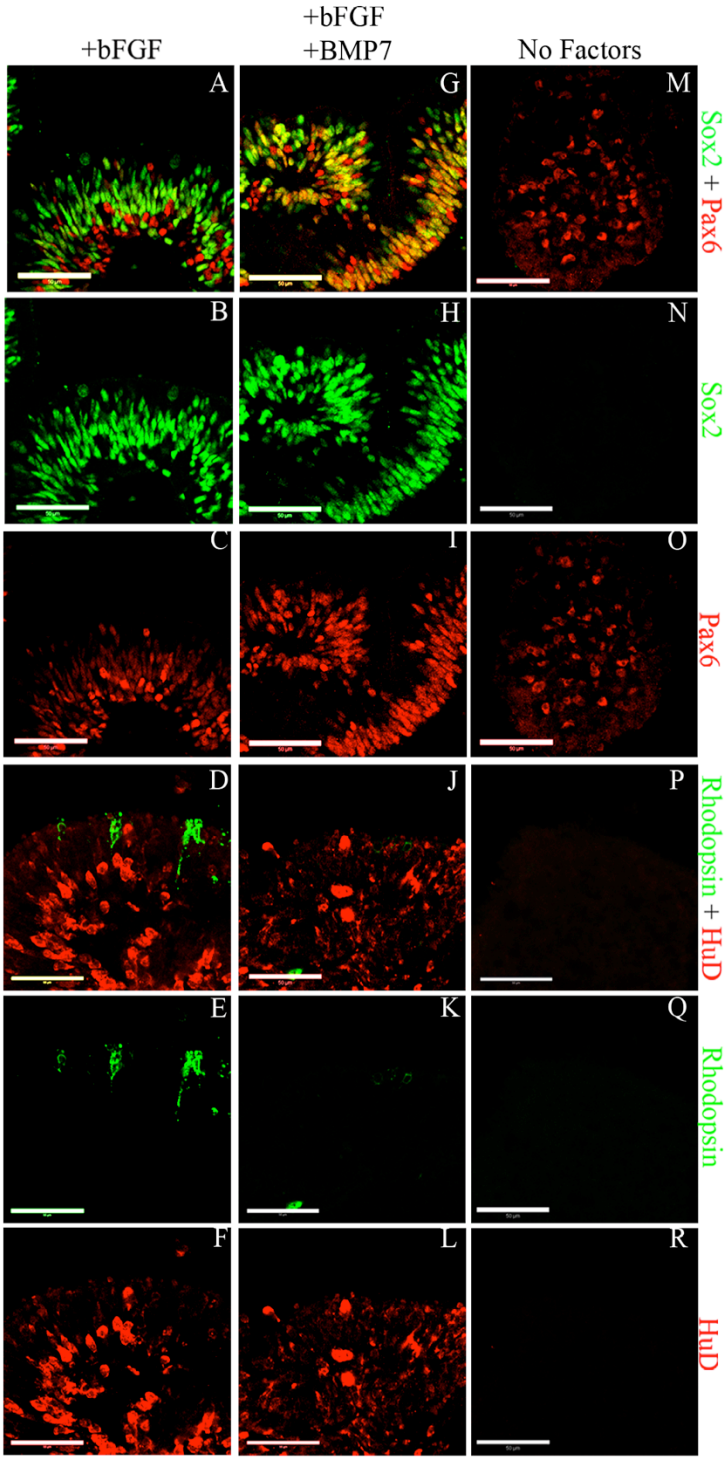


Fig. 4.8i

BMP7 (200ng/ml) does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture.

RPE treated with +bFGF (A-C, J-L) and +bFGF+BMP7 (D-F, M-O) both displayed similar characteristics of transdifferentiation through expression of retinal markers, Pax6 (A, C and D, F respectively), Sox2 (A, B and D, E respectively), HuD (J, L and M, O respectively) and rhodopsin (E, E and J, K respectively) in a neuroepithelial pattern. The localisation of rhodopsin and HuD suggest an inverted retinal phenotype which is characteristic of transdifferentiated retina. Untreated controls did not express retinal markers is most instances (G-R), except some untreated explants did express Pax6 (O). Fluoresence digitally enhanced. Error bars: standard error. Scale bars: 50um.

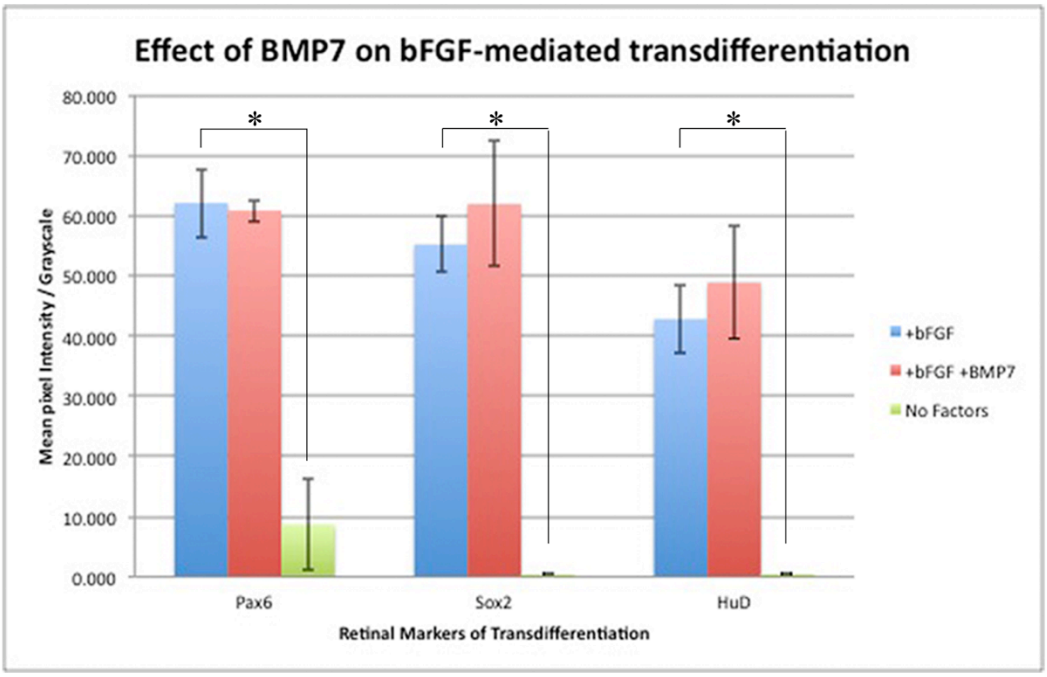


Fig. 4.8ii

Fluoresence quantification of retinal marker expression in chick RPE transdifferentiation assay to observe the effect of BMP7 (200ng/ml) of bFGF (100ng/ml) mediated transdifferentiation.

BMP7 does not inhibit the expression of retinal markers Pax6, Sox2 or HuD in transdifferentiated retina, given that the expression of these markers is comparable in both +bFGF-treated (Blue bars) or +bFGF/+BMP7-treated (Red bars) culture conditions. However, cells treated with bFGF displayed significantly higher expression of all retinal markers in comparison with untreated controls (Green bars)($p<0.05$, RANOVA, $n = 3$). Error bars: Standard error.

fluorescence intensity for Pax6 expression confirmed the lack of significant difference between the +bFGF and +bFGF+BMP7 conditions (Fig. 4.8ii). However, the untreated condition exhibited significantly less expression of the transcription factor than both conditions containing bFGF. This would suggest that BMP7 does not inhibit the onset of transdifferentiation, and does not effect the expression of Pax6 in retinal development. This expression pattern for Pax6 across 3 conditions was also observed to be statistically significant (Fig. 4.8ii, RANOVA, $p<0.05$, $n=3$). The average expression of Sox2 was found to be slightly higher in +bFGF+BMP7 treated explants than +bFGF only explants, however, once again this difference was not observed to be significant. In contrast, the lack of Sox2 expression in untreated explants was found to be considerable less than in the growth factor treated explants, as expected from the images in Fig. 4.8iM-N. This expression pattern between the 3 culture conditions was once again found to be statistically significant (Fig. 4.8ii, RANOVA, $p<0.05$, $n=3$). The expression of HuD in +bFGF+BMP7 treated explants appeared to be slightly higher in intensity than that of +bFGF alone explants, however, once again, image analysis showed that this difference was not significant (Fig. 4.8ii). Both growth factor treated conditions displayed much higher expression of HuD than untreated controls in which the expression was absent. This expression pattern across the 3 culture conditions was again observed to be statistically significant (Fig. 4.8ii, RANOVA, $p<0.05$, $n=3$).

In addition to the expression of retinal markers of RPE transdifferentiation being present in explants treated with both bFGF and bFGF+BMP7, RPE in both of these culture conditions was observed to lose most, if not all, pigmentation in comparison to untreated controls (Fig. 4.9A-C). Depigmentation is a classical characteristic of RPE cells undergoing transdifferentiation towards a retinal phenotype (see Chapter 1) in response to bFGF treatment. In addition to the lack of pigmentation, the morphology of these regions resembled the developing retinal neuroepithelia found in both developing eyes, and transdifferentiating retinas (Fig. 4.9A, B). These neuroepithelia did contain very few pigment granules, reflecting their previous specification as an RPE monolayer, however, the level of pigmentation in these cultures were significantly less than untreated controls, which retained a heavily pigmented

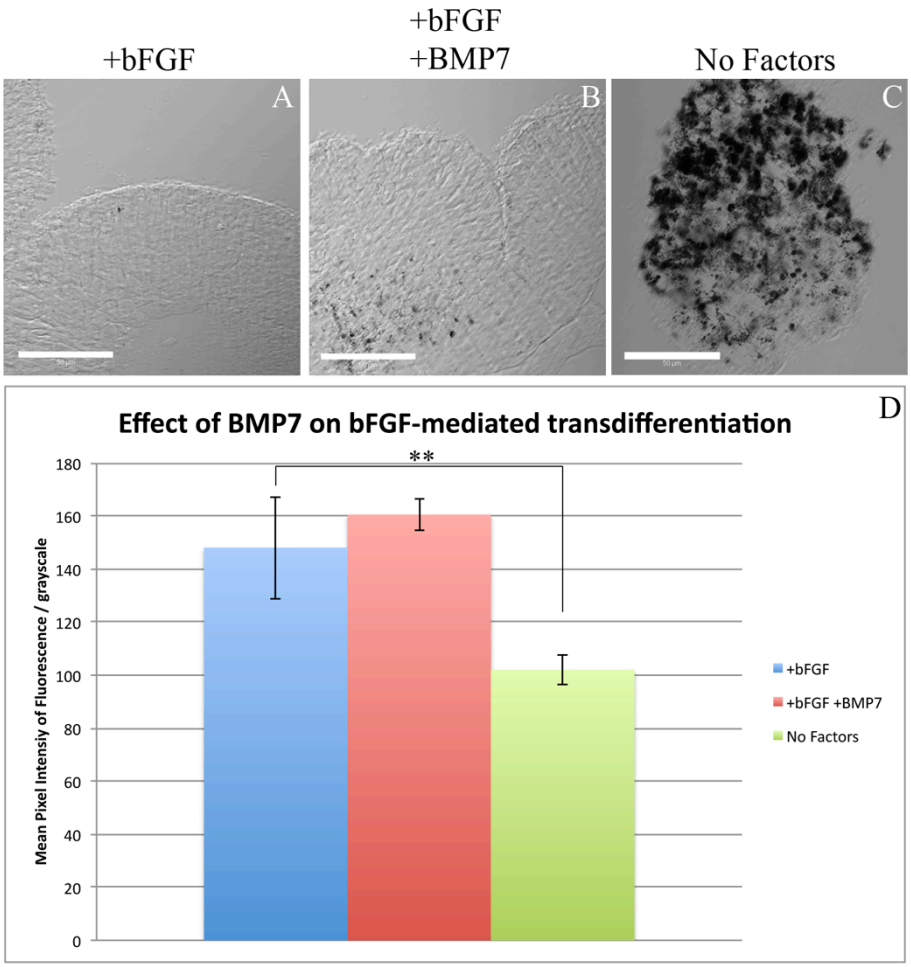


Fig. 4.9
BMP7 (200ng/ml) does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture.
bFGF-treated (Blue bars), and bFGF/BMP7-treated (Red bars) explants displayed comparable level of pigmentation, which implies that BMP7 does not inhibit bFGF-mediated transdifferentiation. RPE explants treated with +bFGF (A) and +bFGF+BMP7 (B) both displayed significant decrease in the level of pigmentation when compared with untreated controls (Green bars) (C). This is a classical characteristic of RPE to neural retina transdifferentiation which indicates that BMP7 does not inhibit bFGF-mediated onset of transdifferentiation. The graph shows the relative levels of pigmentation of explants in each culture condition after 7 days. This was quantified using image analysis software which gave the average pixel intensity of each pixel, in a selected area of the image. This selected area encompassed the whole tissue region only for each image analysed. No background was included. Untreated explants are significantly more pigmentated than growth factor treated explants. Error bars: standard error. Scale bars: 50um (** $p<0.01$, RANOVA, $n = 3$).

RPE phenotype (Fig. 4.9C). This was confirmed by image analysis of the explants which indicated that the average level of pigmentation of +bFGF+BMP7 treated explants was slightly less than that of +bFGF controls, however, standard error bars show that this difference is not significant (Fig. 4.9D). As expected, the level of pigmentation in untreated controls was observed to be significantly more than transdifferentiated RPE explants in the other 2 conditions. This pattern of pigmentation was shown to be statistically significant across the 3 culture conditions (Fig. 4.9D, $p<0.01$, $n=3$).

4.2.3.3 BMP5:

RPE explants treated with both bFGF+BMP5 also exhibited characteristic marker expression suggesting transdifferentiation had taken place (data not shown), with expression of Pax6, Sox2, HuD and rhodopsin present in the explants cultured in this condition, whereas untreated, negative controls did not express retinal markers. This would suggest that BMP5, like BMP4 and BMP7, does not inhibit the onset of transdifferentiation in response to bFGF treatment. However, the levels of marker expression were not consistent for this assay and therefore, it is difficult to reliably quantify the differences between the treated conditions and untreated conditions with regard to these markers. Despite this, RPE explants treated with both +bFGF and +bFGF+BMP5 both displayed a loss of pigmentation, with the +bFGF+BMP5 condition exhibiting slightly less pigmentation than the +bFGF control (Fig. 4.10A-C). Quantification of the level of pigmentation in explants of each condition showed that, while there was a decrease in pigmentation in +bFGF+BMP5 compared with +bFGF only, this difference was not significant (Fig. 4.10D). The de-pigmented areas in +bFGF and +bFGF+BMP5 treated cells closely resembled the columnar, neuroepithelial characteristics associated with transdifferentiated RPE cells (Fig. 4.10A, B). These areas did retain some pigmentation but it was much less intense than the untreated controls. The fact that some pigmentation is retained in these retina-like regions is perhaps evidence of a previous specification as an RPE monolayer. Untreated negative controls were shown to contain

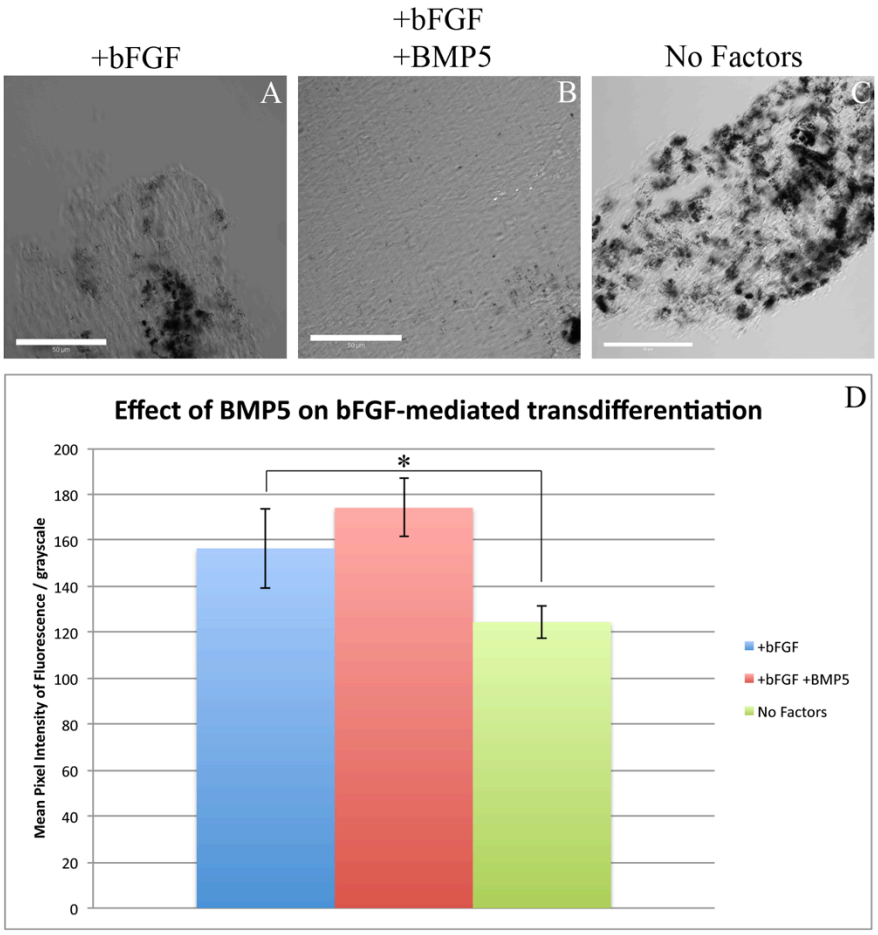


Fig. 4.10
BMP5 (200ng/ml) does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture.
BMP5 treated explants are still observed to undergo the bFGF-mediated, classical di-pigmentation and formation of a neuroepithelium associated with RPE transdifferentiation, in a comparable manner to bFGF only control (D). RPE explants treated with +bFGF (Blue bars) (A) and +bFGF+BMP5 (Red bars) (B) both displayed significant decrease in the level of pigmentation when compared with untreated controls (Green bars) (C). The graph shows the relative levels of pigmentation of explants in each culture condition after 7 days. This was quantified using image analysis software which gave the average pixel intensity of each pixel, in a selected area of the image. This selected area encompassed the whole tissue region only for each image analysed. No background was included. Untreated explants are significantly more pigmented than growth factor treated explants. Grayscale: 0 = Black, 255 = White. Error bars: standard error. Scale bars: 50um (* $p<0.05$, RANOVA, n = 3).

significantly more pigmentation than both the growth factor treated culture conditions, and this pattern of pigmentation was shown to be statistically significant across the three culture conditions (Fig. 4.10D, $p<0.05$, $n=3$).

4.2.4 Discussion:

4.2.4.1 *BMP7:*

Chick RPE HH24 explants, which are able to reliably transdifferentiate towards a retinal phenotype when treated with bFGF for 7 days, were treated with a high concentration of BMP growth factors, BMP4, BMP5 and BMP7, in order to see if the activation of these signaling factors are able to inhibit the onset of transdifferentiation. BMP7 did not appear to inhibit the transdifferentiation of RPE in response to bFGF, as explants treated with both BMP7+bFGF were observed to express retinal markers Pax6, Sox2, rhodopsin, and HuD at comparable levels the a +bFGF positive control. The expression of these markers in both +bFGF and +BMP7+bFGF treated conditions was shown to be significantly higher than in untreated RPE explants, as one would expect of RPE cells which have undergone transdifferentiation, when compared with those which had not. Again unsurprisingly, this pattern of expression level across the three conditions was shown to be statistically significant for each of the retinal markers. The difference between the expression of retinal markers between +bFGF treated, and +bFGF+BMP7 treated explants was not statistically significant, which supports the fact that BMP7 at this concentration, in this *in vitro* assay, is not able to inhibit bFGF-mediated transdifferentiation. Additionally, it would suggest that BMP7 does not effect the expression levels of Pax6, Sox2, and HuD during the early stages of retinal development. The neuroepithelium resulting from transdifferentiation in both growth factor treated conditions contained a large number of Pax6 and Sox2 positive retinal progenitors, which indicates the immature state of the novel retina, however, many of the most intensely labeled Pax6 positive cells were not expressing Sox2. Most of these Pax6 positive cells were

localized to the basal surface of the neuroepithelium, which corresponds to the developing ganglion cell layer in inverted, transdifferentiated retinas. Therefore, it is likely that these cells have down-regulated Sox2 expression in order to differentiate towards specific retinal cell types, most likely ganglion cells in this instance, given that they are the first cells type to be born in developing retina (Prada et al., 1992, Prada et al., 1991, Kahn, 1973) and maintain Pax6 expression in development (Nishina et al., 1999). Some of these highly Pax6 positive cells in the middle of the neuroepithelial layer could possibly be differentiating amacrine cells, which closely follow ganglion cell birth in the developing retina (Prada et al., 1992, Prada et al., 1991). However, it is possible that these cells are also be differentiating ganglion cells that are in the process of migrating towards the basal surface (Bovolenta et al., 1997). Further evidence of the inverted nature of transdifferentiated retina in the two growth factor-treated conditions was the presence of rhodopsin-positive cells at the apical surface of the neuroepithelium. This is where the developing photoreceptors would be expected to be found. Expression of cell membrane localized rhodopsin, in cells which display neuronal like process, would suggest that the few cells which are observed to express rhodopsin are the first rods born during retinal development. Similarly, on some occasions, HuD expression was largely localized to the basal, ganglion cell layer, with some cells also expressing the protein throughout the middle of the neuroepithelium. However, the localization of this protein was observed to be much more variable than other markers. Its expression was observed throughout the neuroepithelium in both +bFGF and +bFGF+BMP7 treated conditions. However, no consistent differences in the pattern of marker localization was apparent between these two conditions, and any differences were most likely as a result of the variability of the assay. The reason for the variability of the assay is unclear, however, given the fact that a complex array of signaling networks are employed during normal retinal development, and retinas resulting from transdifferentiation *in vitro* have a disorganized conformation, it is likely that the relevant signaling gradients, which normally specify the localized expression of retinal markers, will be effected. Therefore, a level of variability of expression level, and localization of retinal markers is to be expected. Similarly, another

source of variability may be the size and specific shape of the initial RPE explants. Naturally, these variables were kept as consistent as possible within each of the cultures, however, given the difficult nature of working with very small, fragile RPE sheets, it is possible that slight variations could also contribute to the level of variability of marker expression. Finally, as described in chapter 3.2, it is perfectly possible that the health of the tissue may have been compromised by 7 days in culture, which would undoubtedly lead to variability in the expression of retinal markers, as a result of disrupted development. Nevertheless, the fact that BMP7 cannot inhibit the onset of transdifferentiation is clear.

4.2.4.2 BMP4:

BMP4 application displayed similar results to BMP7 in that explants treated with +bFGF+BMP4 were observed to undergo transdifferentiation towards a retinal phenotype, like +bFGF only controls. There was no significant difference between the expression levels of Pax6, Sox2 and HuD in +bFGF treated explants compared with +bFGF+BMP4 treated explants. As expected, transdifferentiated RPE did display significantly higher levels of retinal markers than untreated explants, which highlights the fact that these cells retained an RPE phenotype. The variability in the assay is particularly apparent in this data given the relatively large size of the standard error bars on the graph. The source of this variability is unclear, however, some of the reasons previously discussed may be responsible. Interestingly, although not significant, there was a difference in the average expression of Pax6, Sox2 and HuD between the two conditions exhibiting transdifferentiation. Explants treated with +BMP4+bFGF displayed slightly lower expression of retinal markers than +bFGF only positive controls. It is possible that this indicates that BMP4 may have an effect on the expression level of retinal markers, which would become significant at higher doses. However, this does not imply that BMP4 would be able to inhibit bFGF-mediated transdifferentiation at higher doses. The possibility that BMP4 may expression levels of Pax6, Sox2, HuD at higher concentrations may, however, be unlikely, given that these markers are

expressed in different cell types, at different stages of retinal development, and therefore, very unlikely that BMP4 would be specifically involved in regulating the expression of all three genes to a similar degree. Therefore, the observe differences likely result from the variability of the assay as previously discussed. The fact that the pattern of expression for each of these markers was not found to be statistically significant, despite clear transdifferentiation taking place, clearly highlights the effect that the low sample size and variability of the assay has on the statistical significance. Larger sample sizes would most likely be needed in order to confirm the statistical significance of these expression patterns.

4.2.4.3 BMP5:

The expression levels of retinal markers in the assay testing the capacity of BMP5 to inhibit bFGF-mediated transdifferentiation were found to be so variable as to be not consistent enough to quantify, reliably. Nonetheless, both +bFGF treated controls and +bFGF+BMP5 treated explants were both observed to undergo transdifferentiation with characteristic expression of retinal markers Pax6, Sox2 and HuD. These markers were absent in untreated, untransdifferentiated controls. Once again this would suggest that BMP5 cannot directly inhibit bFGF-mediated transdifferentiation *in vitro*.

4.2.4.4 General discussion:

All three BMP growth factors: BMP4, BMP5 and BMP7, did not appear to display inhibitory properties for bFGF-mediated transdifferentiation. This was further highlighted by the fact that the levels of pigmentation between both growth factor treated conditions, +bFGF and +bFGF+BMP were not significantly different, and were observed to have significantly less pigmentation than untreated controls. Therefore, given the fact that de-pigmentation is a major characteristic of transdifferentiated RPE cells, this evidence, in conjunction with the expression of retinal markers associated with transdifferentiated retina, strongly suggests that

even in the presence of each of the three BMP growth factors, transdifferentiation is still able to take place.

At this juncture it is important to note the limitations of the image analysis technique used to quantify the level of expression of various markers, in addition to the level of pigmentation of the tissue. The selection of the weakest fluorescence signal was performed by approximation manually, which means that there is potential bias and error in the quantification of the overall average pixel intensity. This is compounded by the fact that the images used are often relatively small regions of tissue at high magnification, some of which appear to display very healthy, consistent expression of markers and pigmentation, whereas others less so. This again introduces variability/error into the assay given that some images contain more cells with less intensity than other regions of the same tissue might exhibit. Additionally, this assay does not account for the slight variation in background signal which may be seen between different sample slides, and therefore, this is likely to once again introduce error. In addition to the practical application of this particular image analysis technique, it is also assumed that the level of fluorescence intensity directly correlates to the level of expression of a particular marker, which, although likely would, may not be reflect the precise expression level of a labeled protein. It is possible that batch effects may arise as a result of processing a large quantity of tissue at the same time, which causes some samples to be more amenable to fluorescent antibody labeling, whereas others less so. This could mean that, although a good indicator of the relative level of expression of different markers, this assay may not provide a precise measurement of the level of expression on a particular retinal marker. The fact that quantification was performed using grayscale images would also limit the sensitivity of the assay, because a pixel can only be given a non-continuous value between 0 and 255, so values are rounded to the nearest integer for each pixel, rather than measured on a continuous scale which accounts for more minor variations in intensity. Despite this, the relatively large size of the grayscale, in addition to the large number of overall pixels from which the average pixel intensity is calculated, should render this loss in sensitivity negligible.

Taking into account that BMP signaling factors do not appear to have the capacity to directly regulate Pax6 expression in RPE explants, and that they do not have the capacity to inhibit bFGF-mediated transdifferentiation *in vitro*, this begs the question as to how BMP signaling factors are able to augment to RPE phenotype during development (Muller et al., 2007). Given the outcome of this investigation, the fact that no obvious effects of BMP signaling directly on the RPE were exhibited, they would imply that BMPs maintain the RPE phenotype indirectly, through secondary signaling mechanisms. Indeed it has been shown that the transdifferentiation of the RPE following ectopic application of noggin to the developing optic cup, correlates with an increase in the expression of FGF8 in the central retina (Adler and Belecky-Adams, 2002). Ectopic application of FGF8 to the developing optic cup, in a similar manner to other FGFs, is able to induce RPE to neural retina transdifferentiation (Vogel-Hopker et al., 2000). Therefore, an increase in the concentration of FGF8 in the central retina, immediately adjacent to the RPE monolayer, could cause a shift in the demarcating line in the concentration gradient between FGFs from the surface ectoderm, and TGFβ/activin signaling from the mesenchyme, which specifies whether the multi-potent cells of the optic cup become neural retina or RPE cells respectively (Fig. 4.12) (Hyer et al., 1998, Muller et al., 2007, Fuhrmann et al., 2000b, Araki et al., 1998, Araki et al., 2002, Pittack et al., 1997, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993, Fuhrmann, 2010, Sakami et al., 2008, Guillemot and Cepko, 1992, Nguyen and Arnheiter, 2000). Normal expression of FGF8 in the retina would most likely not induce transdifferentiation for the RPE owing to the antagonistic effects of other signaling mechanisms which are able to block a fate switch towards neural retina, in much the same way that activin has been reported to do (Sakami et al., 2008). However, it is reasonable to suggest that an increase in the effective dose of FGF8 immediately adjacent to the RPE, in response to a loss in BMP signaling, would be able to overcome the effects of these inhibitory mechanisms, and thus initiate transdifferentiation of the RPE. The implication therefore is

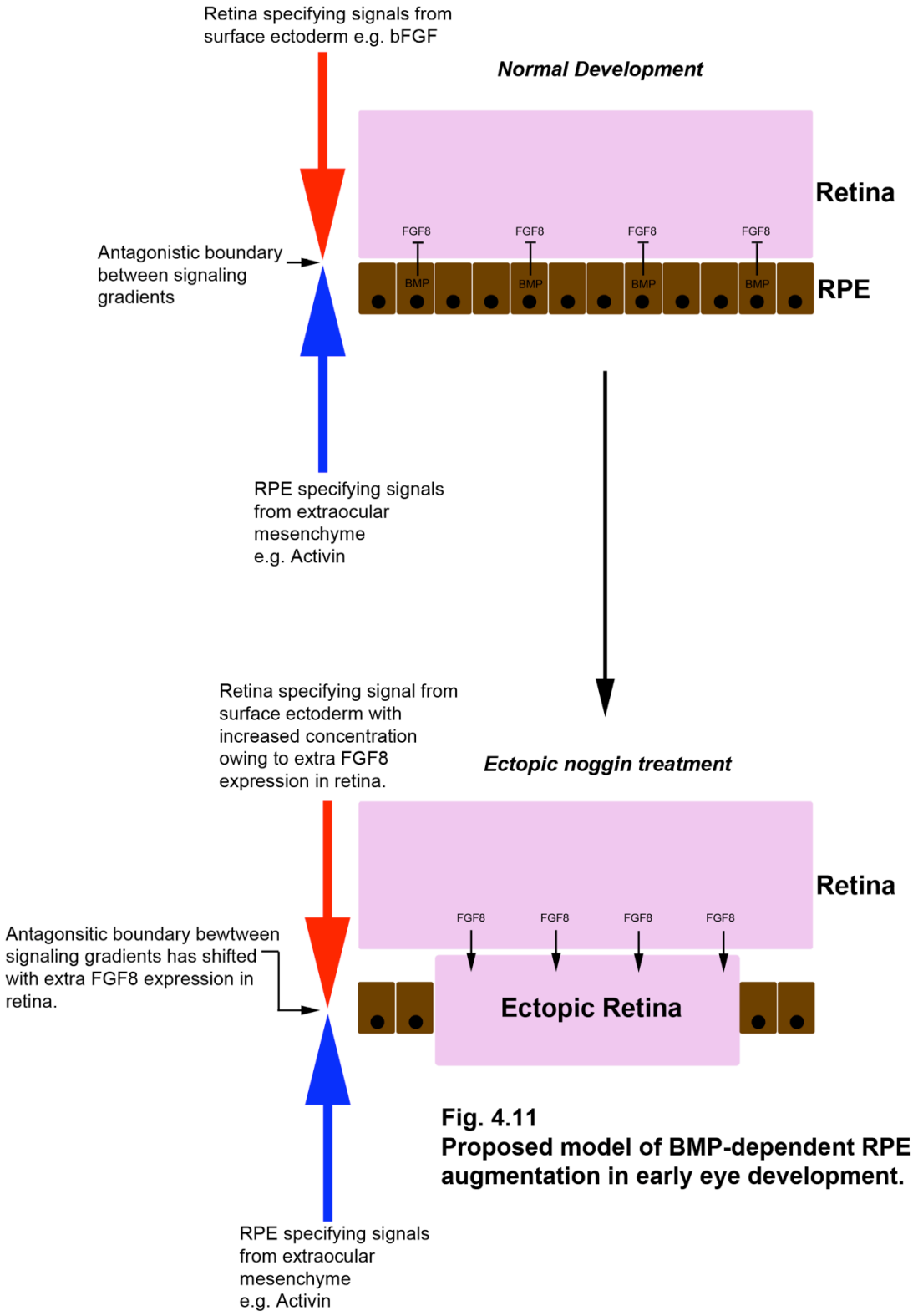


Fig. 4.11
Proposed model of BMP-dependent RPE augmentation in early eye development.

that BMP signaling is able to act indirectly to inhibit FGF expression in the retina, much like it has been reported to in the development of the head (Ohkubo et al., 2002, Crossley et al., 2001). By doing so, BMP signaling from the extraocular mesenchyme and RPE cells (Muller et al., 2007) appears to be able to act as a protective boundary for the RPE, limiting the effects of FGF signaling required for the proper development of the retina, and helping to maintain the RPE phenotype. This model of a concentration-dependent gradient in the development of the bipotent optic cup is consistent with the classical understanding of retinal development. Indeed, the data exhibited in the report by Muller et al (Muller et al., 2007) shows inhibition of BMP signaling via ectopic noggin application, which resulted in transdifferentiation of the entire RPE monolayer, except for the most dorsal portion of the RPE which largely retained its phenotype. This untransdifferentiated region is in contact with the over-lying dorsal mesenchyme reported to be the region with the highest expression of BMPs, which is consistent with its reported role in dorsal patterning of the retina (Zhang and Yang, 2001). Therefore, this region is likely to have a higher localized concentration of BMPs which would be able to inhibit the noggin-mediated increase of FGF8 expression in the adjacent retina, unlike distal retina, and thus inhibit transdifferentiation from taking place. This model is also consistent with reports that ectopic applciation of BMPs are able to cause transdifferentiation of the neural retina towards an RPE phenotype in a dose-dependent manner. Ectopic BMPs could possibly inhibit the expression of FGF8 and perhaps other FGFs required for the specification and differentitation of the retina, which in turn would down-regulate the Chx10 expression responsible for neural retina specification (Horsford et al., 2005, Nguyen and Arnheiter, 2000). This would result in a subsequent increase in Mitf expression, which induces RPE-like characterisitics such as pigmentation.

Further experiments would be necessary in order to confirm this model, however the existing evidence certainly supports this hypothesis. Future experiments would require the co-implantation of both Noggin and anti-FGF blocking antibody/pharmacological inhibitor of FGF to observe whether or not the reported noggin-mediated transdifferentiation of the RPE was inhibited.

4.3 Can exogenous Shh inhibit bFGF-mediated transdifferentiation of chick RPE HH24?:

4.3.1 Introduction:

Given the fact that BMP growth factor signaling members did not appear to inhibit the onset of transdifferentiation in response to bFGF, another growth factor, Shh, which has been implicated in the augmentation of the RPE phenotype, as well as the inhibition of bFGF-mediated transdifferentiation (Spence et al., 2004, Perron et al., 2003, Zhang and Yang, 2001, Spence et al., 2007a), was tested using the same assay.

4.3.2 Materials & Methods:

All methods were the same as in the previous experiment, except the BMP signaling factors were substituted for Shh (Invitrogen) (200ng/ml). Additionally, as well as the standard 100ng/ml dose of bFGF, the experiment was also repeated using a lower dose of bFGF of 50ng/ml.

4.3.3 Results:

The expression levels of the retinal markers Pax6, Sox2, HuD and rhodopsin were very variable in both +bFGF and +bFGF+Shh culture conditions, which made reliable quantification difficult (data not shown). However, once again the RPE explants were observed to lose their characteristic pigmentation in response to bFGF in both +bFGF+Shh and bFGF only treatments (Fig. 4.12A-C), in comparison with untreated controls. De-pigmented regions were observed to retain a low level of pigmentation throughout the neuroepithelial structures, which indicated their previous identity as RPE cells (Fig. 4.12A, B). Quantitative image analysis of the levels of pigmentation in each condition confirmed that both +bFGF and +bFGF+Shh conditions contained comparable levels of pigmentation

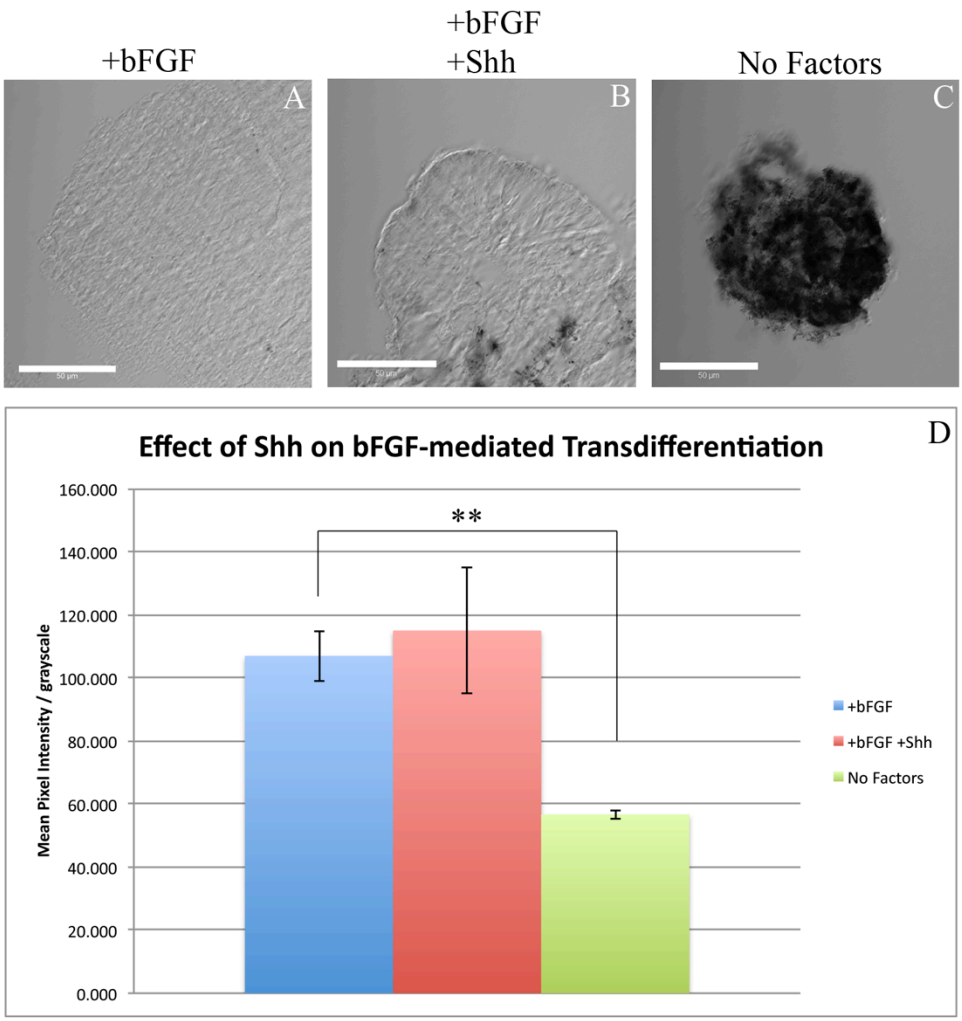


Fig. 4.12
Shh (200ng/ml) does not appear to inhibit bFGF-mediated transdifferentiation of HH24 RPE after 7 days in non-adherent culture.
RPE explants treated with +bFGF (Blue bars) (A) and +bFGF+Shh (Red bars) (B) both displayed significant decrease in the level of pigmentation when compared with untreated controls (Green bars) (C). This is a classical characteristic of RPE to neural retina transdifferentiation which indicates that Shh does not inhibit bFGF-mediated onset of transdifferentiation. The graph shows the relative levels of pigmentation of explants in each culture condition after 7 days. This was quantified using image analysis software which gave the average pixel intensity of each pixel, in a selected area of the image. This selected area encompassed the whole tissue region only for each image analysed. No background was included. Untreated explants are significantly more pigmentated than growth factor treated explants. Grayscale: 0 = Black, 255 = White. Error bars: standard error. Scale bars: 50um (** $p<0.01$, RANOVA, n = 3).

(Fig. 4.12D), however, the untreated condition contained significantly more pigmentation than the growth factor-treated controls. This pattern of pigmentation was also found to be statistically significant (Fig. 4.12D, $p<0.01$, $n=3$).

The evidence for Shh augmentation of the RPE phenotype, in addition to its apparent ability to inhibit bFGF-mediated transdifferentiation of RPE cells early in development, is relatively well established in the literature. Therefore, it was thought that the lack of inhibition of transdifferentiation in response to bFGF in this assay might result from the fact that a relatively high concentration of bFGF (100ng/ml as used in this assay), may be able to overcome the inhibitory effects of Shh, even at high concentrations of 200ng/ml Shh. It was therefore necessary to test a lower dose of bFGF, with the same high dose of Shh, in order to see if Shh can inhibit the transdifferentiation-inducing effects of bFGF under these conditions. The assay was repeated with the same concentration of Shh (200ng/ml), but the concentration of bFGF was lowered to 50ng/ml. RPE explants treated with 50ng/ml bFGF + Shh still resulted in transdifferentiation in a similar manner to the higher does of bFGF (data not shown). It is possible that the inhibitory effects of Shh on transdifferentiation are very dose sensitive, which is why no inhibition was observed in these assays, however, on current evidence, Shh has not been shown to inhibit bFGF-mediated transdifferentiation *in vitro*.

4.3.4 Discussion:

A high dose of Shh (200ng/ml) was not observed to inhibit bFGF mediated transdifferentiation in isolated explants as evidenced by the formation of de-pigmented neuroepithelial loops in cultures treated with both +bFGF and +bFGF+Shh when compared with untreated controls, which maintained their pigmented RPE phenotype as expected. This pattern of pigmentation across the three conditions was observed to be highly significant, as one would expect when comparing heavily pigmented cells, with those that have lost their pigmentation in response to treatment. There was no significant difference between the

+bFGF positive control condition and the +bFGF+Shh condition, which suggests that Shh does not inhibit de-pigmentation as a result of transdifferentiation of the RPE. Neuroepithelia resulting from treatment with growth factors both expressed retinal markers Pax6, Sox2, HuD and rhodopsin as expected, and these markers were absent from all untreated explants, except for a small number which appeared to retain some Pax6 expression as previously discussed. However, the variability of the assay (as discussed previously) once again made reliable quantification of the specific expression levels of each marker difficult, as they were particularly inconsistent. It has been reported that high concentrations of Shh expression in the retina can lead to cell death, which may have been a contributing factor to this variability (Spence et al., 2004, Perron et al., 2003, Zhang and Yang, 2001). Shh has been reported to have direct effects on the capacity for transdifferentiation of RPE cells, therefore it was thought that perhaps the relatively high dose of bFGF used was enough to overcome a 200ng/ml dose of Shh. This was because Shh has been reported to antagonize bFGF-mediated RPE transdifferentiation when constitutively expressed in RPE, except in regions which are immediately adjacent to a bFGF-coated bead, where bFGF concentration is highest (Spence et al., 2007a). This would suggest that at high concentrations of bFGF, Shh is ineffective in inhibition of transdifferentiation. Therefore, a lower concentration of bFGF (50ng/ml) was employed in the same assay. RPE still underwent transdifferentiation when treated with both +bFGF (50ng/ml)+Shh (200ng/ml) in a similar manner to that of +bFGF (50ng/ml) treated explants only, which may suggest that a relative dose effect of bFGF vs. Shh is not responsible for the apparent inability of Shh to inhibit transdifferentiation. It will be necessary to examine the effects of different doses of Shh in future investigations, however, given the reported apoptosis-inducing effects of Shh at high concentrations, it may be that increasing the concentration any higher than that used in this investigation, may simply lead to more retinal cell death, rather than an increased capacity for inhibition of transdifferentiation. It was hoped that inhibition of Shh signaling in RPE cells post-HH24 (when the potential for transdifferentiation in response to bFGF begins to be lost), using a pharmacological inhibitor called cyclopamine, would forgo the need for testing several doses of Shh in future studies,

and demonstrate whether these explants could transdifferentiate more readily in response to bFGF than those untreated with the drug. However, this drug was found to be particularly toxic to RPE explants, even when employed at very low concentrations *in vitro*, and therefore no meaningful data could be obtained (data not shown).

It is possible that given the above evidence, Shh does not directly signal to the RPE, but instead utilizeses an indirect mechanism in order to maintain the RPE phenotype, in a similar manner to that proposed for BMP signaling in the development and augmentation of the RPE. This hypothesis is supported by the fact that Shh inhibition *in vivo* was only reported to initiate RPE transdifferentiation in eyes with an intact retina (Zhang and Yang, 2001). Cylopamine-treatment of retinectomized eyes had no effect on the RPE phenotype (Spence et al., 2004) which suggests that inhibition of Shh in the intact eye is once again able to up-regulate a secondary signaling factor originating in the retina that can subsequently induce transdifferentiation. It is possible that, like inhibition of BMP signaling, inhibition of Shh could lead to an increase in the expression of FGFs in the retina which then induces transdifferentiation of the RPE. However this has yet to be confirmed. It is an attractive hypothesis, but it does not account for several other findings which have also been reported. Implantation of bFGF-coated beads in addition to cyclopamine, was shown to potentiate the effects of bFGF, and increase the domain of transdifferentiation without the presence of the native retina (following retinectomy) (Spence et al., 2004). It is possible the the presence a regenerating retina at the ciliary marginal zone is able to respond to Shh and up-regulate FGF expression, which may account for this expansion of the domain of transdifferentiation, via an increase in the dose of FGF reaching the RPE. However, this model does not account for the fact that ectopic expression of Shh in intact eyes appears to be able to antagonise FGF-mediated activation of a its dowstream effector, ERK, via phosphorylation (Spence et al., 2004). The labeling of pERK does not appear to be increased in comparison to bFGF controls, when RPE is treated with both bFGF and cyclopamine. These data would suggest that ectopic expression of Shh in the retina may be able to activate the expression of an unknown factor which is able to antagonize FGF signaling. If this factor can be identified, it may therefore be

a good candiate for the restriction of the capacity for transdifferentiation. It would be interesting to test whether the effect of ectopic Shh expression on phosphorylation of ERK in the RPE is retained following retinectomy, or in explanted RPE cultures. Taken together with the results of this investigation, one would not expect Shh to have the same effect in the absence of adjancent tissues.

The sonic hedgehog pathway and its effectors therefore remain an intriguing prospect with regards to the potential restricition of the capacity for transdifferentiation. More investigation of this and other pathways will be necessary in order to ascertain the limiting factor for transdifferentitation. It may be that, as suggested by Sakami et al (Sakami et al., 2008), epigenetic changes in the RPE cells which occur with on-going development, eventually restrict changes in the phenotype of the RPE cell. This is another avenue which will require investigation.

4.4 To what extent can embryonic chick RPE explants HH24 intrinsically down-regulate Pax6 expression in culture?:

4.4.1 Introduction:

Explanted RPE sheets were observed to undergo a down-regulation of Pax6 regardless of the treatment with exogenous growth factors, after at least 2 days in culture. In order to ascertain the capacity of the RPE to intrinsically down-regulate Pax6 over time in culture, RPE explants were prepared and cultured without growth factor treatment and sampled at a number of different time points. If RPE cells are able to down-regulate the expression of Pax6 without the presence of other optic tissues, or exogenous growth factors, this would suggest that some mechanism within the RPE cells themselves exists which leads to this phenomenon. Given the strong implication of a maintenance of Pax6 expression in the retention of the capacity for transdifferentiation (Spence et al., 2007b, Kuriyama et al., 2009b,

Azuma et al., 2005a), this mechanism could also be important for controlling the loss of capacity for transdifferentiation as the RPE develops.

4.4.2 Materials & Methods:

Embryonic chick RPE explants HH24 were isolated as described in chapter 2, and cultured as in experiment 4.1, except without any exogenous growth factor treatment. Immunohistochemistry was performed as described in chapter 2.

4.4.3 Results:

HH24 stage RPE sheets were shown to robustly express Pax6 in all cells across the explant in post-dissection controls, fixed immediately following dissection (Fig. 4.13A-C). As in previous experiments, Pax6 was maintained in all the RPE cells after just one day in culture (Fig. 4.13D-L), despite the fact that the RPE in this assay was developmentally older (by approximately 1 day) than the HH21 RPE used in the previous assay. Similarly, the level of pigmentation was also found to be variable after 1 day in culture (Fig. 4.13 F, I) with the darkest pigmentation generally confined to the centre of the RPE explant. However, after 2 days in culture, the RPE explants began to resemble the varied level of Pax6 expression across the sheet, previously observed in 2-day cultures of HH21 RPE (Fig. 4.13K, N). Some large areas of the sheet, in this case largely restricted to the centre of the explant, appeared to have a very low expression of Pax6, compared with the periphery of the explant. It was possible that this observation had resulted as an artifact of the cells lying in different visual planes, however, careful analysis of multiple visual planes using a confocal microscope, as well as the even, nuclear labeling of the DAPI in the same visual pane, both suggest that this is not the case (Fig. 4.13 J, M). All the cells are lying in the same visual plane.

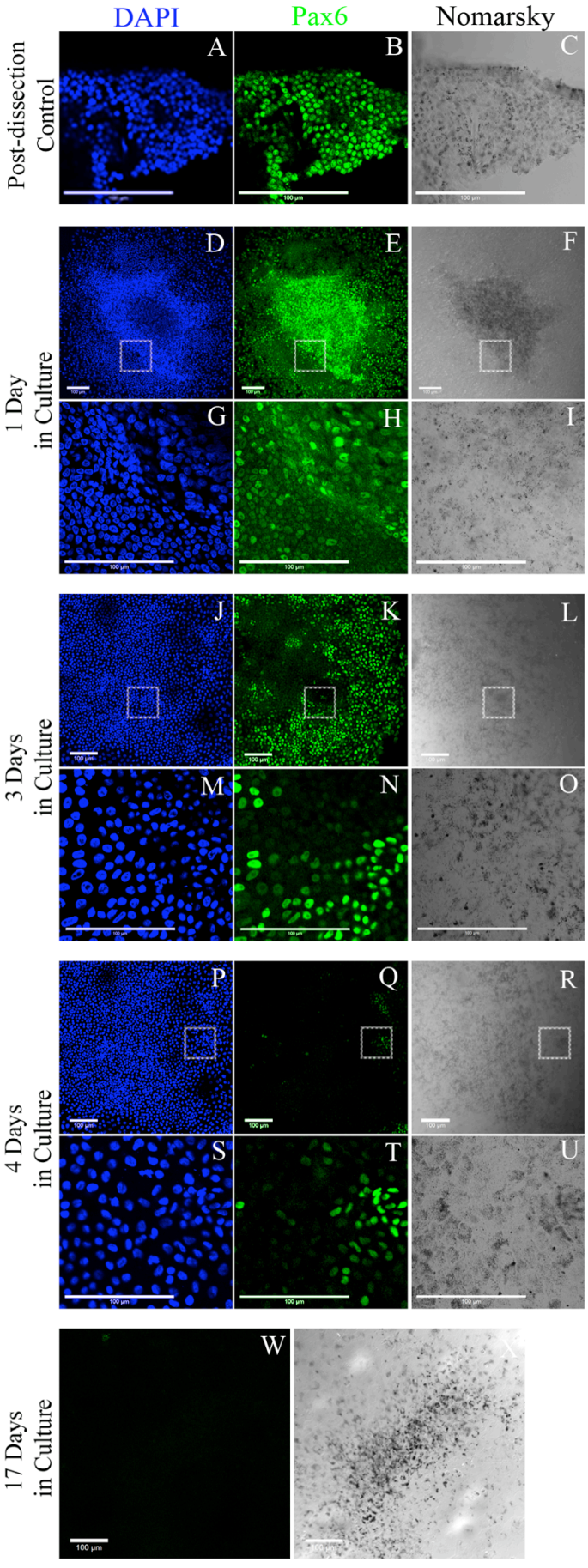


Fig. 4.13
Explanted HH24 chick RPE intrinsically loses Pax6 expression over time in culture. Pax6 (green) was expressed in all cells after 1 day in monolayer culture (E, H - white box high mag.), however, by 3 days in culture, the level of expression of Pax6 was observed to become variable, with large regions of the monolayer having down-regulated Pax6 (K, N - white box high mag.). By 4 days in culture, the majority of explanted RPE cells had intrinsically lost the expression of Pax6, with only a small cluster of cells retaining the expression of the transcription factor (Q, T - White box high mag.). No Pax6 expression was observed in explanted RPE after 17 days in culture (W). DAPI (Blue). Scale bars: 100uM.

The level of Pax6 expression did not appear to correlate with the level of pigmentation of the RPE explant given that the same, general level of pigmentation was consistent across the whole explant. After 4 days in culture, nearly all the cells of the RPE explant have levels of Pax6 expression, which are barely above the background (Fig. 4.13Q, T). Once again all the cells lie in the same visual plane as evidenced by the consistent DAPI labeling across the sheet (Fig. 4.13P, S) and the level of Pax6 expression does not appear to correlate with the level of pigmentation. Only a couple of small patches of cells continue to express relatively high levels of Pax6 after 4 days in culture (Fig. 4.13Q white box, T). Explants left for a number of days more were sampled at 17 days in culture, where no detectable Pax6 expression was present in the RPE sheets (Fig. 4.13V). This suggests that RPE cells may be able to intrinsically down-regulate their own expression of Pax6, without the need for other tissues. The mechanism by which the cells are able to do this remains unknown.

4.4.4 Discussion:

Explanted RPE cells did display an intrinsic ability to progressively down-regulate Pax6 expression across the monolayer, over a number of days in culture. The clustering of cells with low Pax6 into distinct regions, may suggest that the down-regulation of Pax6 is caused by a local, micro-environmental effect. It is possible that RPE cells in this region are themselves able to intrinsically down-regulate Pax6 expression via the release of short-range, autocrine/paracrine signals, which are only able to effect cells which are immediately adjacent to themselves. These regions with low Pax6 expression are observed to become larger with time in culture, until eventually, very few RPE cells retain robust expression of Pax6 after 4 days in culture, and no cells express the transcription factor by 17 days in culture at the latest. This gradual decline in Pax6 expression may imply that isolated RPE cells, at least to some degree, can continue their maturation *in* vitro. This trend over time in culture may support the idea of autocrine signaling factors being present, as these factors would be able to gradually spread throughout the entire explant culture given enough time. It is possible that a feedback

loop could exist which would allow cells receiving the signal to down-regulate Pax6, to themselves pass on the message via expression of the same autocrine/paracrine signal. This idea would correlate with the idea that the RPE sheet does not develop *en masse* as a single, developmentally-equivalent sheet of cells, but instead develops and matures more regionally (see chapter 3). However, at present, no conclusive data exists to prove this hypothesis. It was thought that the cells with a lower expression of Pax6 might be developmentally more mature than those still expressing high levels of the transcription factor, given that it is observed to be down-regulated as the RPE mature (see Chapter 3)(Spence et al., 2007b).

If the intrinsic ability of RPE explants to down-regulate Pax6 expression does involve a diffusible signaling factor, the identity of this molecule(s) is yet to be identified. However, chick RPE cells of a comparable developmental stage to those used in this investigation have been reported to express the TGFβ-signaling family member, activin, as well as its corresponding receptor machinery, at gradually increasing levels with development (Sakami et al., 2008). Activin has been reported to down-regulate the expression of Pax6 in RPE cells, as well as antagonize bFGF-mediated transdifferentiation *in vitro* (Sakami et al., 2008, Fuhrmann et al., 2000b). This suggests that activin could act as an autocrine, signaling molecule which acts directly on isolated RPE cell explants in culture, and in doing so contribute to the down-regulation of Pax6. In addition, it has been reported that inhibition of activin signaling is able to extend to window of competence for bFGF-induced transdifferentiation of RPE cells, which may result, given the apparent need for maintenance of Pax6 expression for a maintenance in the potential for transdifferentiation (Sakami et al., 2008), via anatomization of its reported action in down-regulation of Pax6, and augmentation of the RPE phenotype (Fuhrmann et al., 2000b). In future experiments, it will be necessary to analyse the capacity of activin inhibition in maintenance of Pax6 expression in explant chick RPE cultures. Additionally, given that these effects of activin inhibition are reported to be limited to a particular developmental stage (HH29), which is still very early in embryonic development, it is likely that other candidates that regulate the expression of Pax6, and

therefore restriction of transdifferentiation, exist. These may include other signaling pathways, as well as epigenetic and chromatin structure changes.

Interestingly, cultures at HH21 and HH24 both exhibited similar levels of Pax6 down-regulation after 2/3 days respectively, despite the former developmental stage being approximately 1 day earlier than the latter. Could this suggest the existence of an *in vivo* signal that maintains the expression of Pax6 in RPE cells isolated later in development? Possible candidates would include FGFs from the adjacent retina (Vogel-Hopker et al., 2000), which have been shown to up-regulate and maintain Pax6 expression in RPE cells (Spence et al., 2007b, Kuriyama et al., 2009a). If such a factor were to exist, it is possible that RPE cells are only able to down-regulate Pax6 expression once isolated for a particular period of time, approximately 2 days in the current culture system. Perhaps more likely is that a period of lag phase is required for isolated RPE explants to produce a threshold level of an autocrine/paracrine signal (should this exist) for it to be effective.

Chapter 5 –

Human fetal development and transdifferentiation studies

5.0 Introduction

The capacity for transdifferentiation of RPE cells towards neural retina is most likely conserved in a number of different species. This feature of RPE cells appears to be similar to that of multi-potent retinal progenitor cells of the optic cup, which are able to specify both the RPE and the neural retina in early development (Pittack et al., 1997, Coulombre, 1981, Coulombre and Coulombre, 1965, Coulombre and Coulombre, 1970, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993, Zhao et al., 1995, Sakami et al., 2008). It is likely, given the conservation of the capacity for transdifferentiation at the very earliest stages of RPE development in non-urodele species, that human RPE cells at a similar stage would be able to undergo a similar process. Consistent with this hypothesis, it is also likely that the capacity for transdifferentiation in human RPE cells would also become restricted at later stages of development. It will therefore be necessary to investigate the potential for transdifferentiation in the most developmentally immature human RPE cells available.

5.1 Human fetal eye developmental study:

5.1.1 Introduction:

In order to have a baseline expression profile for human RPE cells at different stages of human development, the earliest available fetal eye tissue was investigated using immunohistochemical analysis to ascertain the spatio-temporal expression patterns of various retinal and RPE markers. This also served to ratify the specificity of the antibodies which were to be used in further human RPE and retinal studies.

5.1.2 Methods & Materials:

Human embryonic tissue of several developmental stages was obtained from the Human Developmental Biological Resource bank (Institute of Child Health, Gt. Ormond Street Hospital, London) and eye globes were mechanically removed from the rest of the head using fine, watch-makers' forceps. Some of these were subsequently fixed in 4% paraformaldehyde overnight for Immunohistochemical analysis. Eye globes were often damaged so only a select few eyes were in suitable condition for Immunohistochemical analysis.

Immunohistochemical analysis was performed as described in chapter 2.4.

5.1.3 Results:

A summary of the expression of each marker at each developmental stage is displayed in table 5.1.

Initially, the expression patterns of 2 markers often associated with RPE cells, Pmel17, a marker of developing melanocyte lineage, and bestrophin1, a protein which is largely regarded as being specifically expressed in RPE cells, and is crucial for RPE function later in development, were investigated. Bestrophin1 expression was analysed in both central and peripheral RPE cells, adjacent to the optic nerve head, and in the region often referred to as the ciliary marginal zone (CMZ), respectively. CS18 human fetal eye tissue was positively labelled for the expression of bestrophin1 in lightly pigmented RPE cells adjacent to the retina (Fig. 5.1i A-C yellow arrows). The fluorescent signal was low in intensity which would suggest that the level of expression of bestrophin1 in the RPE is low at this stage. Some cells appeared to express the protein at higher levels than others. Crucially, the pattern of expression was localized to the baso-lateral surface of the cells, which is consistent with the location of a cell membrane bound protein. Similarly, bestrophin1 was also expressed in the CMZ region of CS18 human fetal RPE cells, which also displayed apical pigmentation (Fig.

Developmental Stage	N number	Pmel17		Bestrophin		Nestin		Pax6	
		Central	Peripheral	Central	Peripheral	Central	Peripheral	Central	Peripheral
CS18	2	+	+	+	+	+	+	+	+
CS23	3	+	+	+	+	+	+	NA	NA
F1	4	+	+	NA	NA	+	+	+	NA
F2	2	+	+	-	+	NA	NA	+	+
F3	1	+	+	-	NA	+	NA	+	+

Table 5.1
A summary of the expression of retinal markers at different stages of fetal human eye development

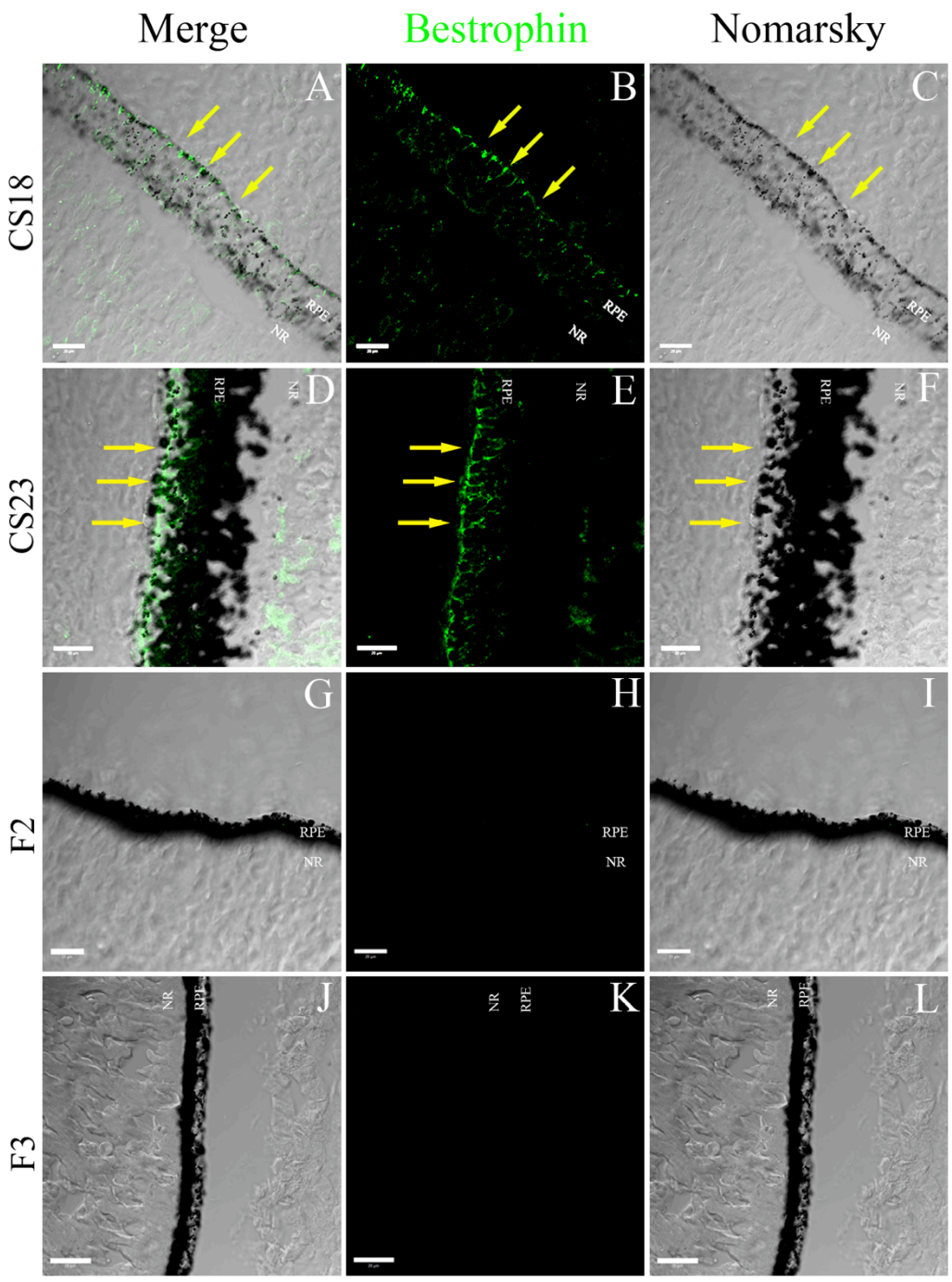


Fig. 5.1i
Bestrophin1 expression in developing human fetal retina stages CS18-F3 - Central retina.
Bestrophin1 (green) was only detected in pigmented RPE at the earliest stages of retina analysed, CS18 (A-C) and CS23 (D-F). The pattern of bestrophin1 expression was characteristically baso-lateral in appearance which is consistent with expected distribution (A-F, yellow arrows). No expression was observed in the RPE at later stages F2 (H) or F3 (K). Bestrophin1 expression was not observed in the neural retina at any stage (B, E, H, K). Scale bars: 20uM.

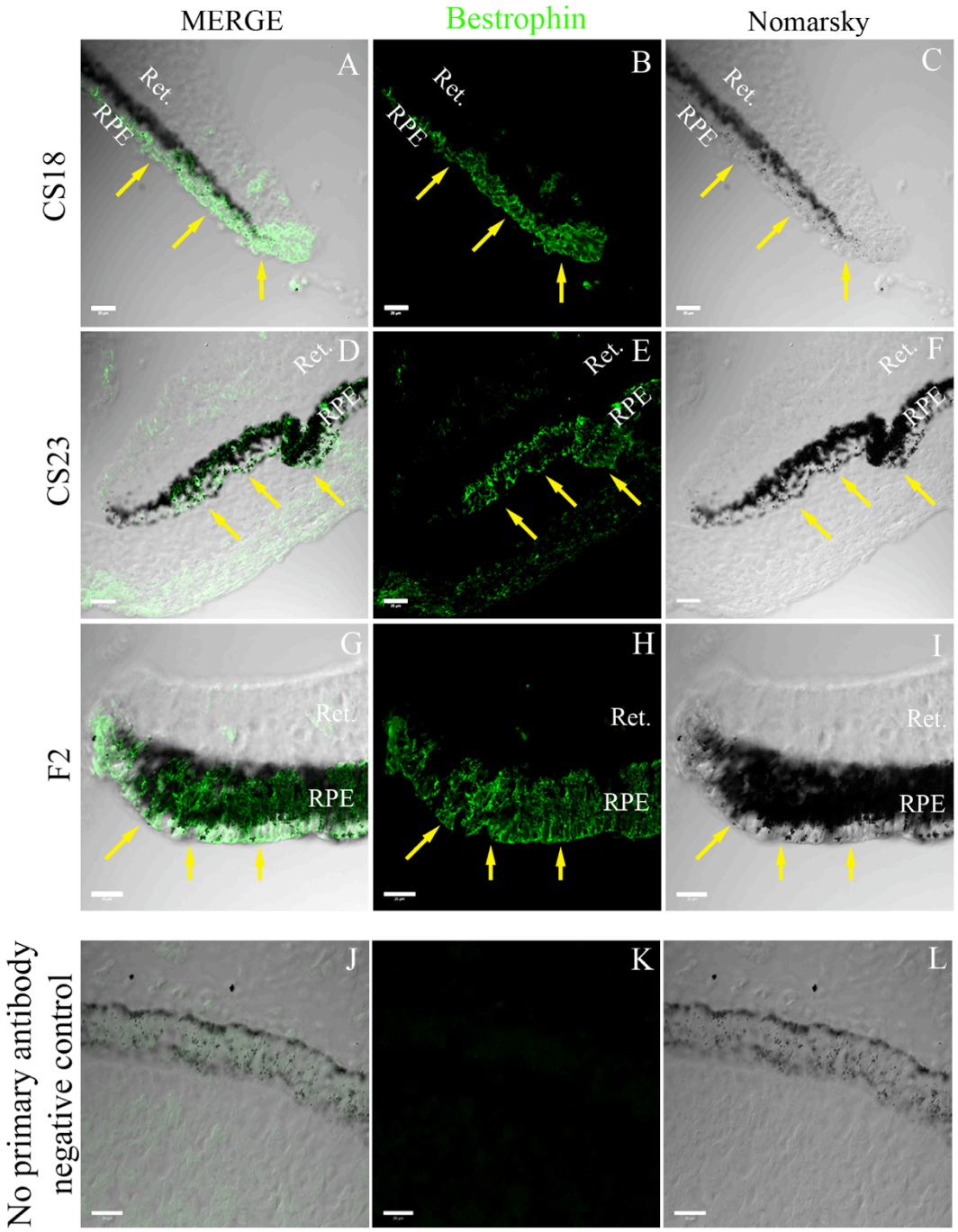


Fig. 5.1ii

Bestrophin1 expression in the developing human fetal retina stages CS18 to F2 - periperhal retina.

Bestrophin1 was observed to be expressed in the periperhal, pigmented RPE at all stages of development analysed: CS18 (A-C), CS23 (D-F), F2 (G-H). the pattern of expression was characteristically baso-lateral as expected (yellow arrows), with the majority of neural retinal cells observed to be negative for expression of bestrophin1 (B, E, H). However, at the earliest stage of development, CS18, some non-pigmented cells at the RPE-retina junction were also observed to express some bestrophin1 (A). This expression pattern was not observed in the non-pigmented cells in the corresponding regions at later developmental stages: CS23 (D), or F2 (G). No primary antibody control contained no signal in human fetal retina (J-L). Scale bars: 20uM.

5.1iiA-C yellow arrows). The expression pattern of bestrophin1 in peripheral RPE cells appeared to be most strongly labelled at the baso-lateral surface of the cells, as with central RPE, however, peripheral cells appeared to display labelling at low levels at the apical surface, as well as in the cytoplasm (Fig. 5.1iiA-C). It is possible that this signal is background fluorescence, which appears to be high in these regions. Interestingly, bestrophin1 labelling appears to extend into the presumptive retina at the boundary between pigmented RPE cells, and non-pigmented retinal cells. This region displays very little bestrophin1 signal despite very little pigmentation being present. The expression of bestrophin1 in CS23 human fetal eyes is more robust and even than that of CS18 (Fig. 5.1iD-F yellow arrows), and is still largely localised to the baso-lateral surface of the heavily pigmented RPE cells. No bestrophin1 expression is found in the non-pigmented presumptive retina in this central region, with only a low level of auto-fluorescence present in some damaged tissue in the retina (Fig. 5.1iE). Again, bestrophin1 expression in the CMZ of CS23 tissue was only confined to the pigmented cells of the RPE (Fig. 5.1iiD-F yellow arrows), and no expression was present in non-pigmented retinal cells, adjacent to the RPE. This included the non-pigmented retinal cells at the border of the presumptive retina and the developing RPE, unlike that observed in CS18 tissue. The RPE at CS23 was much more heavily pigmented in the CMZ (Fig. 5.1iiD, F) than CS18 RPE (Fig. 5.1iiA, C). No positive signal was detected in heavily pigmented RPE cells in F2 human fetal tissue (Fig. 5.1i G-I) or non-pigmented presumptive retinal cells. In contrast to the apparent absence of bestrophin1 expression in central RPE at F2, peripheral RPE cells, located in the CMZ, were observed to be very strongly labelled for basolateral expression of bestrophin1 in heavily pigmented RPE cells (Fig. 5.1iiB, E, H yellow arrows). No positively labelled cells were present in non-pigmented presumptive retinal cells adjacent to bestrophin1 positive RPE cells at this stage. Central RPE cells in F3 human fetal RPE tissue were also found to be absent for the expression of bestrophin1 in pigmented RPE cells (Fig. 5.1iJ-L), as were non-pigmented presumptive retina. The primary negative control was absent for any positive fluorescence, with only a low level

of background signal being detected (Fig. 5.1iiJ-L). This confirms that the signal present in the experimental sections was as a result of specific, primary antibody binding.

Pmel17 has been utilised as a sensitive marker of RPE cells owing to their heavily pigmented phenotype. Once again Pmel17 proved to be a robust marker of RPE cells with the pigmented RPE monolayer at several different developmental stages. RPE monolayers in both the central region of the eye, adjacent to the optic nerve head, and RPE at the RPE-retinal boundary in the CMZ, were positive for Pmel17 expression (Fig. 5.2, 5.3, 5.4i, 5.4ii). In the central RPE, the pigmented cells were labelled in a granular fashion, characteristic of Pmel17 expression, at CS18, CS23, F1, F2 and F3 (Fig. 5.2, 5.4i, 5.4ii). Interestingly, at stages CS23 and F1, some Pmel17 expression was unexpectedly observed in the Pax6 positive, presumptive retina, which was free of pigmentation. (Fig. 5.2D, E, 5.4iG, H, L, M yellow arrows) (n=3). These sections were not immediately adjacent to the optic nerve head, but were found to be more peripheral. The most intensely labelled cells in the retina were immediately adjacent to the Pmel17 positive RPE monolayer, with an observed gradient in the level of Pmel17 expression in the retina, which decreases towards the middle of the presumptive retina. In order to confirm that this expression was indeed expressed in the neural retina, and not a result of a sectioning artefact, it was necessary to co-label Pmel17 with other known retinal and neuronal markers. Pmel17 expression in the non-pigmented retinas of peripheral sections of human fetal eyes at stage CS23 and F1 was co-localized with neuronal marker nestin (Fig. 5.4iF-H, K-M respectively. yellow arrows). Similarly, Pmel17 expression in the peripheral section of F1 tissue was observed to co-localise with Chx10, a transcription factor which is characteristic of undifferentiated retinal neuroepithelium, early in development (Fig. 5iF-H yellow arrows). No Pmel17 expression was observed in the Chx10 positive retinal cells in a more central location (Fig. 5iJ-L). No Pmel17 expression was observed in CS18 sections, or any section investigated at the later stages of development F2, or F3 (Fig. 5.2B-D, P-S, 3G-L). In addition to the expression of Pmel17 observed in the RPE monolayers at all stages, some, non-pigmented, single cells were observed to express Pmel17 in the extra-ocular space at F1 and F2 (Fig. 5.2D,E, G, H white arrows, 5.3K-M, 5.5J,K).

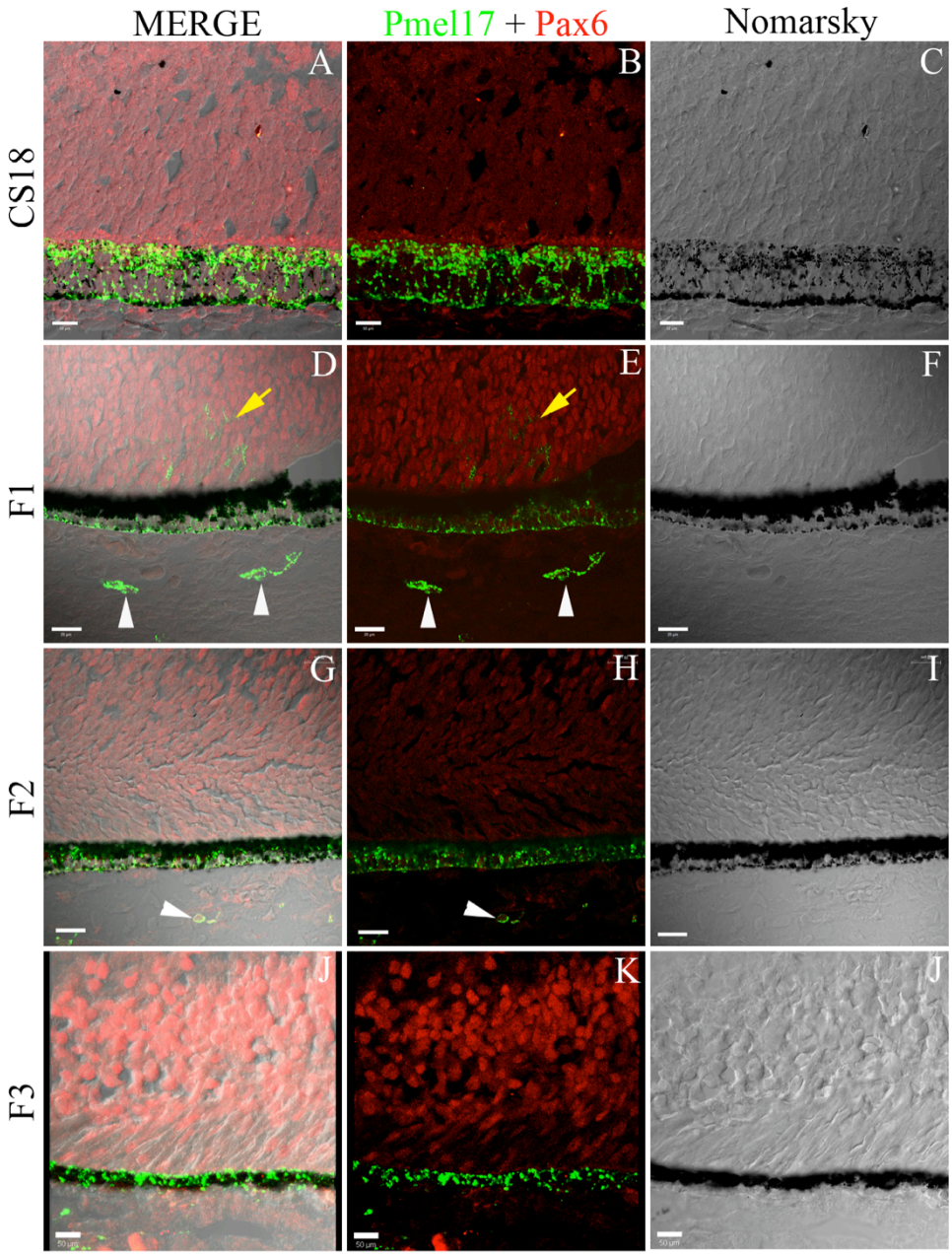


Fig. 5.2
Expression of Pmel17 and Pax6 in developing human fetal retina stages CS18 to F3 - Central retina.
Pax6 (red) expression was observed in the presumtive retina at all stages investigated (A-L), but was absent from pigmented RPE cells at all stages investigated (A-L). Pigmented RPE cells were observed to express Pmel17 at all stages investigated (A-L), and Pmel17 was largely absent from presumptive retina. However, some cells in the extraocular mesenchymal tissue on the basal side of the RPE were also observed to express Pmel17 in some sections (D, E, G, H white arrows). It is unclear as to what the identity of these cells are. Additionally, some cells in a more peripheral section at F1 retina expressed Pmel17 in the Pax6 positive neural retina (D, E yellow arrows) immediately adjacent to the RPE. Scale bars: 20uM (A-C, J-L), 50uM (D-I).

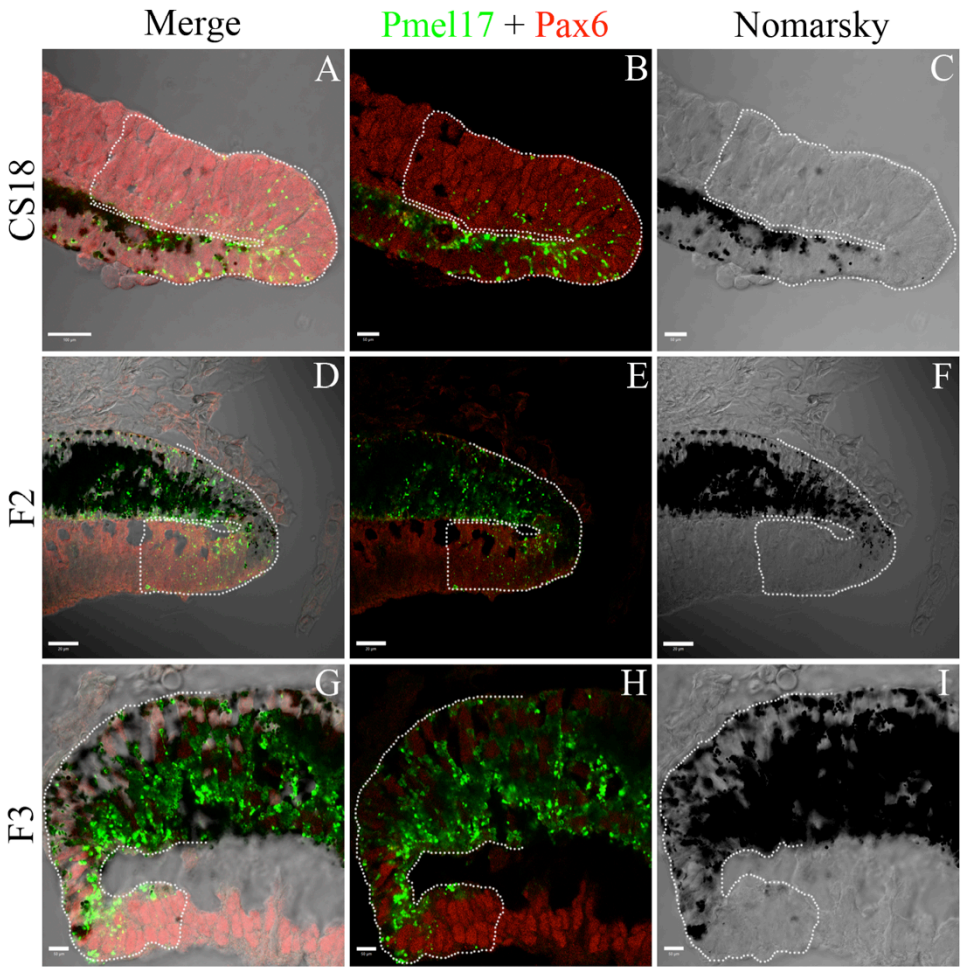


Fig. 5.3
Pmel17 and Pax6 expression in the developing human fetal retina- peripheral retina (CMZ).
Pmel17 (green) expression was observed in pigmented RPE cells at all stages investigated (A, D, G). It was also expressed in Pax6 (red) positive, presumptive retinal cells, which were not pigmented, at all stages: CS18, F2, F3 (B, E, H, respectively, white dotted lines). Pax6 expression was also observed in all cell nuclei comprising the retinal neuroepithelium at all stages (B, E, H), as well as Pmel17 positive, pigmented RPE cells (A, B, D, E, G, H) at all stages investigated. This expression of Pax6 in RPE cells decreased in a peripheral to central gradient across the RPE. Scale bars: 50uM (A-C, G, L), 20uM (D-F).

Pmel17 expression was observed throughout the RPE monolayer at all stages investigated, including at the RPE-retinal margin in the CMZ (Fig. 5.2, 5.4ii, 5.5A-D). The Pmel17 expression appeared to extend beyond the boundary of the pigmented cells, into the non-pigmented, presumptive retina (Fig. 5.2 dotted line, 5.4ii blue arrows, 5.5A-D). The intensity of the expression appeared to be most intense at the pigmented / non-pigmented border, and became gradually reduced the further cells were located into the presumptive retina. The pattern of expression in this region was very sparse and granular, which suggested that most of the Pmel17 expression in this region was expressed at a low level when compared to that in the heavily pigmented RPE cells. These non-pigmented, Pmel17 positive cells were not found to express either neuronal marker nestin (Fig. 5.4iiJ-L), or immature retinal markers Chx10 (Fig. 5.5D), which is unexpected given their apparent specification as developing retinal cells, given their position in the non-pigmented, developing, retinal neuroepithelium.

Pmel17 positive, pigmented, RPE was negative for Pax6 in both central and more peripheral sections at all stages investigated (Fig. 5.2). Presumptive retinal cells that were lacking in pigment expressed Pax6 at all stages investigated. This expression was observed throughout the whole of the developing neuroepithelium, in the nuclei of developing retinal cells, as one would expect. However, in the CMZ, pigmented, Pmel17 positive RPE cells were observed to be positive for Pax6 expression (Fig. 5.3), as were the non-pigmented, presumptive retinal cells adjacent to the RPE. The expression of Pax6 was confined to the nucleus as expected for a transcription factor protein. This was observed to be the case at all stages investigated: CS18, F2 and F3, a broad range of developmental stages for the tissue which was available. The level of expression of Pax6 appeared to be lower in RPE cells at the CMZ boundary, compared with that in the adjacent retina, which was more intensely labelled (Fig. 5.3).

Nestin expression was absent from the Pmel17 positive, pigmented RPE cells at all stages of development investigated: CS18, CS23, F1 and F3 (Fig. 5.4iD, I, N, S). Nestin was however robustly expressed throughout the retinal neuroepithelium (Fig. 5.4iD, I, N, S) in a

fibrillar pattern, perpendicular to the retina, and spanning the breadth of the whole retinal neuroepithelium. This pattern is characteristic of the labelling of neuronal intermediate filaments of the cytoskeleton in immature, nestin positive neurons arranged in a proximo-distal orientation in the retina. The most intense expression of nestin appeared to be localised to the vitreal surface of the presumptive retina, with progressively weaker expression of the protein adjacent to the RPE monolayer, where nestin expression was weakest (Fig. 5.4iD, I, N, S). Nestin was shown to co-localise with Pmel17 in non-pigmented presumptive retinal cells in the retina, as previously discussed (Fig. 5.4iB, G, L, Q). Nestin expression was observed to be absent from the non-pigmented presumptive retinal cells at the far periphery of the CMZ (Fig. 5.4iiJ-L).

The developing retinal transcription factor Chx10, was observed to be absent from Pmel17 positive, pigmented RPE cells in central (adjacent to the optic nerve head) (Fig. 5.5L), peripheral (Fig. 5.5H), and CMZ (Fig. 5.5D) sections of F1 human fetal eye tissue. However, Chx10 was robustly expressed in the nuclei retinal neuroepithelial cells at this stage, with the expression appearing to be restricted to the primary neuroblastic layer (Fig. 5.5 F, H, J, L) adjacent to the RPE, but was absent from the secondary neuroblastic layer present at this developmental stage, which was located at the vitreal surface of the developing retina (Fig. 5.5 F, H, J, L). As previously discussed, no Chx10 expression could be detected in the CMZ of F1 human fetal eye tissue (Fig. 5.5B-D). This included both the Pmel17 positive, pigmented region, in addition to the non-pigmented, presumptive retinal region.

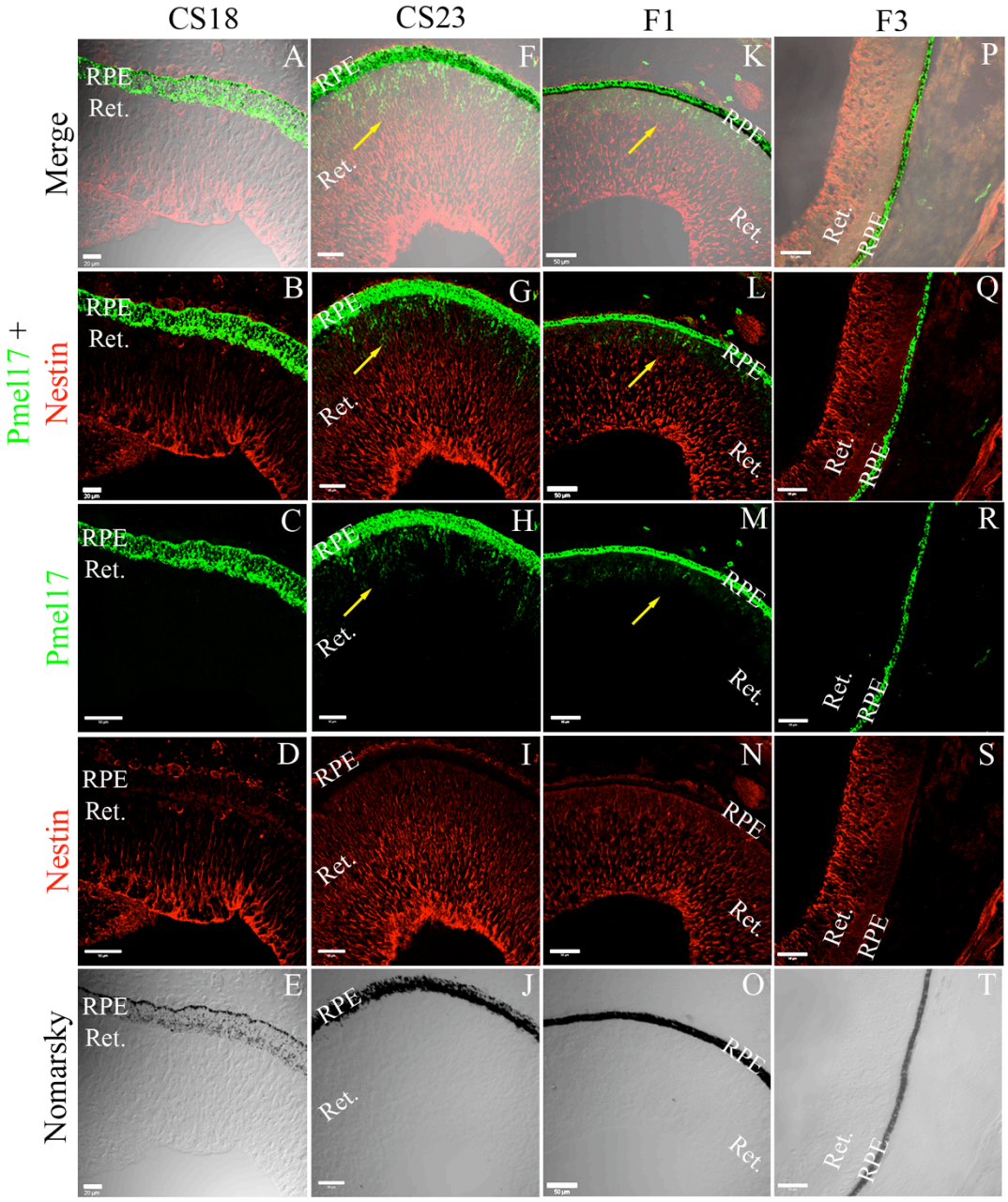


Fig. 5.4i

The expression of Pmel17 and nestin expression in developing human fetal retina - central retina.

Pmel17 (green) was robustly expressed in all pigmented RPE cells, at all stages of development investigated: CS18 (C), CS23 (H), F1 (M), F3 (R). Unexpectedly, Pmel17 expression was also observed in non-pigmented, nestin-positive (red), neuroepithelial cells adjacent to the pigmented RPE at stages CS23 (F, G, H, yellow arrows) and F1 (K, L, M, yellow arrows). This Pmel17 expression was observed in a fibrillar pattern that extended in a gradient towards the middle of the neuroepithelium, with no expression at the vitreal side of the neural retina (H, M). No Pmel17 expression was observed in the neural retina at CS18 (B, C) or F3 (Q, R). Nestin was observed throughout the neural retina at all stages, with the highest expression localised to the vitreal surface, however, nestin was absent from the RPE at all stages (D, I, N, S). Scale bars: 50uM.

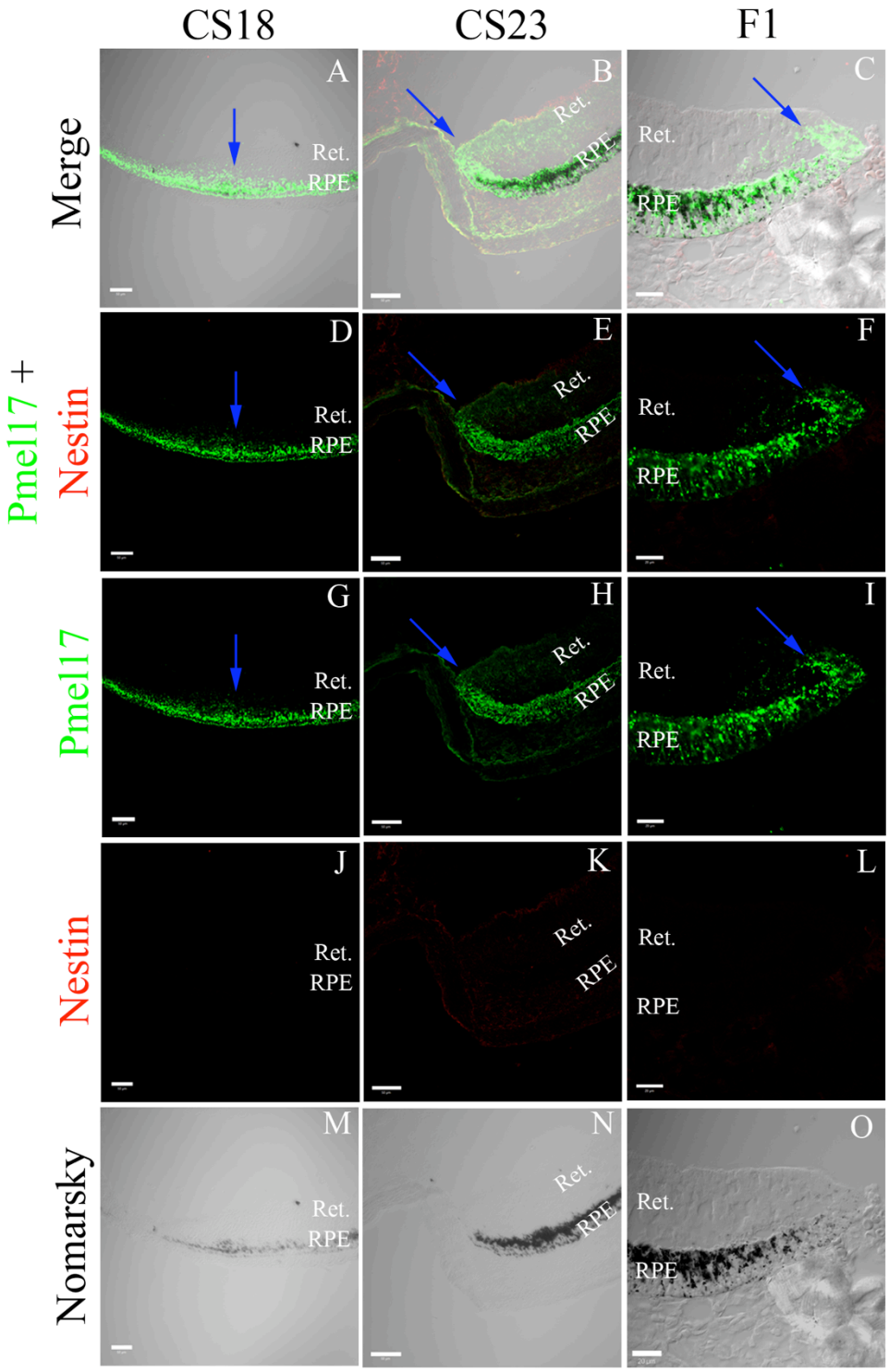


Fig. 5.4ii

Expression of Pmel17 & nestin in the developing human fetal retina at stages CS18 - F1 - peripheral retina.

Peml17 (green) was observed in the pigmented RPE monolayer at all stages investigated: CS18 (A, G), CS23 (B, H), F1 (C, I). Pmel17 was also detected in non-pigmented cells at the RPE-retinal margin at all stages (A-C, G-I, blue arrows). The level of expression of Pmel17 was low in this region and rapidly decreases in a gradient towards the central retina. No nestin (red) expression was observed in either the RPE or presumptive retina at any stage of development in this region (J, K, L). Scale bars: 50uM (A, B, D, E, G, H, J, K, M, N), 20uM (C, F, I, L, O).

Two other transcription factors which are important in eye development are: Otx2, which is critical for RPE development, as well as retinal development, and CRX, which has been associated with retinal development, in particular, photoreceptors. It was therefore necessary to test primary antibodies raised against these proteins because they may need to be used in future experiments. Human fetal eye tissue at developmental stage F1 was observed to express both Otx2 and CRX (Fig. 5.6). Otx2 was expressed in the nuclei of pigmented RPE cells in both central regions adjacent to the optic nerve head (Fig. 5.6A, D), and pigmented cells at the CMZ (Fig. 5.6F, I). It was also expressed in some retinal cells of the presumptive retina, with the most intensely labelled cells residing immediately adjacent to the RPE monolayer, with other positive cells extending throughout the rest of the neuroepithelium in the central region. However, the level of intensity of fluorescence was lower in these cells, suggesting a lower level of expression. In the peripheral CMZ region, Otx2 positive cells were located throughout the breadth of the neuroepithelium, and these cells appeared to exhibit a relatively uniform level of expression (Fig. 5.6F). Similarly, CRX expression was also present in the nuclei of the cells of the developing neuroepithelium, in both central and CMZ regions (Fig. 5.6B, E, G, J). The expression pattern of CRX was very similar to that of Otx2, and in many cells, the two transcription factors appeared to be co-expressed (Fig. 5.6C, H). However, fewer cells were positively labelled for CRX in the outer portion of the neuroepithelium than they were for Otx2, in the central region at this stage. Most of the CRX positive nuclei were located adjacently to the RPE monolayer, which is consistent with the location of future photoreceptor cells following maturation (Fig. 5.6B, E). CRX expression was observed to co-localise with Otx2 positive cells in the peripheral CMZ region, and positive cells spanned the breadth of the neuroepithelium (Fig. 5.6H). Interestingly, CRX expression was present throughout the heavily pigmented RPE monolayer in both the central and CMZ portions of the eye (Fig. 5.6B, E, G, J), which co-localised with Otx2 expression in the RPE.

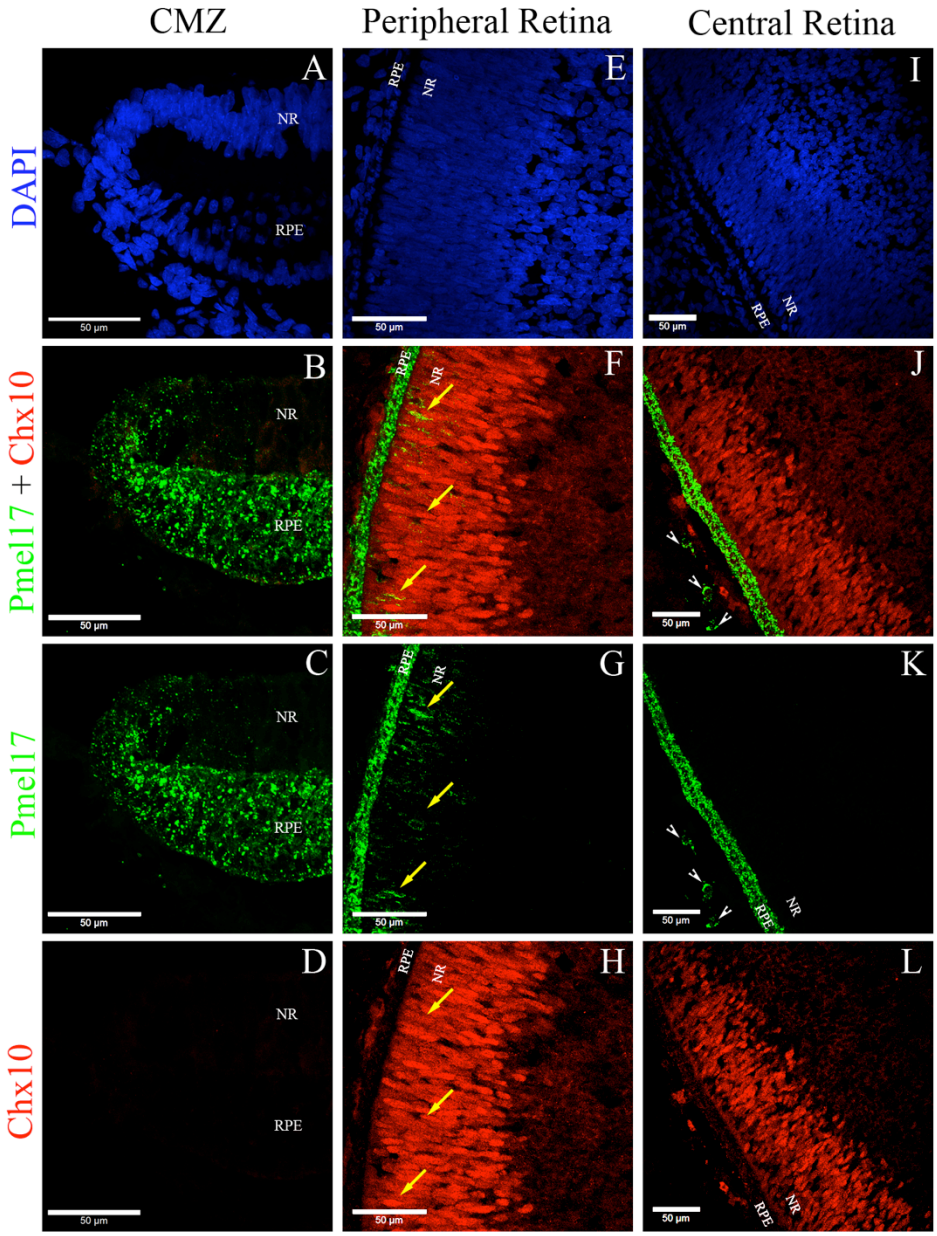


Fig. 5.5
The expression of Pmel17 and Chx10 in human fetal retina at stage F1.
DAPI (blue), Pmel17 (green), Chx10 (red). Pmel17 expresion was observed throughout the pigmented RPE cells in all regions at this stage (C-G). However, in the CMZ the Pmel17 extended into the neuroepithelial region (B, C) which did not express Chx10 (B, C). Pmel17 expression was absent from the presumptive retina in the central retina, adjacent to the optic nerve head (J, K), however,it was observed in the Chx10 positive, presumtpive retina in more peripheral sections of retina (F, G, H yellow arrows). Some Pmel17 was also observed in extraocular mesenchymal tissue (J, K white arrows). Chx10 was confined to the outer neuroblastic layer of the developing retina, but was absent from the inner neuroblastic layer (F, J, H, L), CMZ (B-D) and RPE (A-L). Fluoresence digitally enhanced. Scale bars: 50uM.

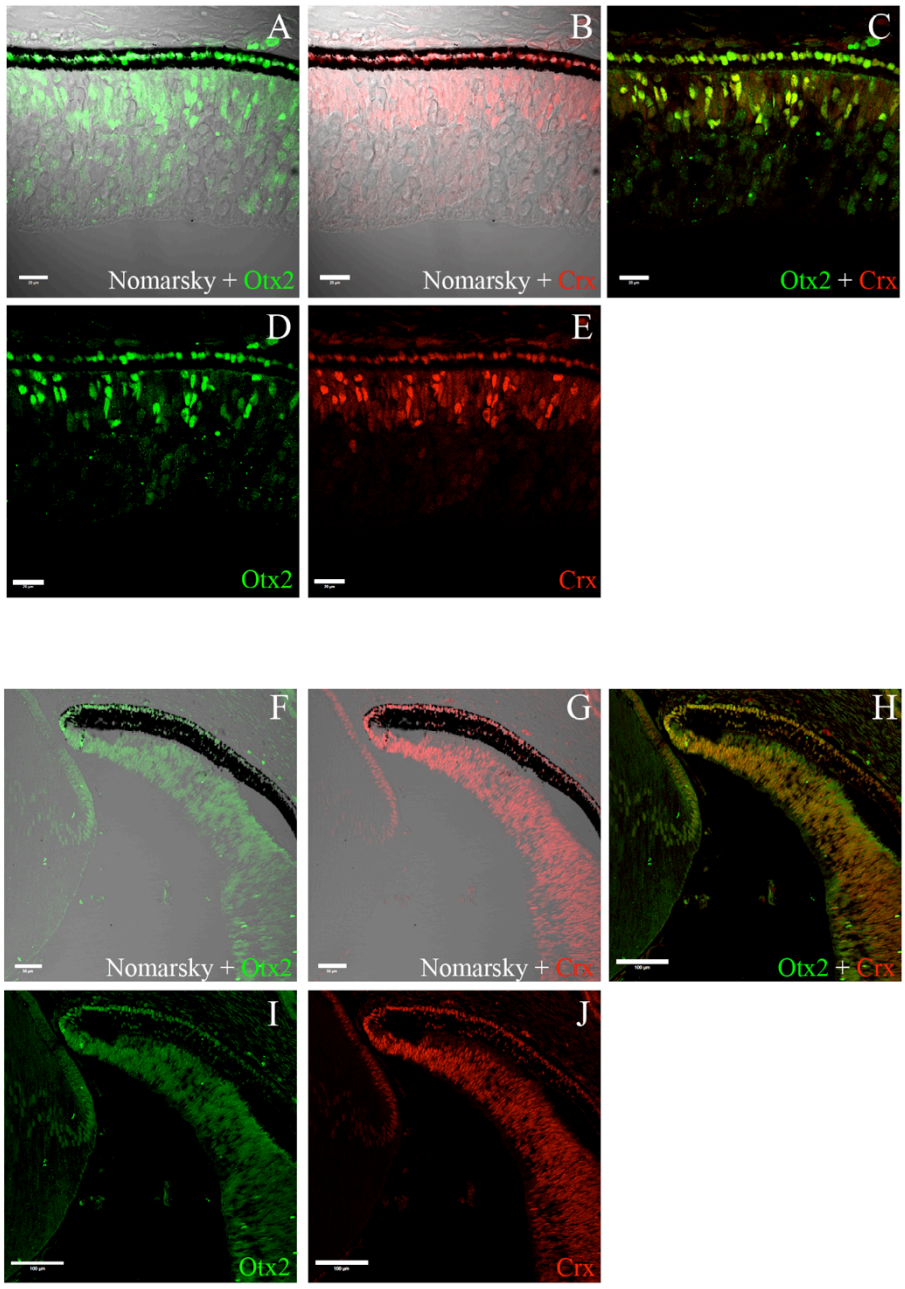


Fig. 5.6

The expression of Otx2 and CRX in developing human fetal retina.

Otx2 expression was most strongly expressed in pigmented RPE cells and presumptive photoreceptors adjacent to the RPE monolayer (A, D) in the central retina. The majority of Otx2 positive cells were also observed to co-express CRX in both central and CMZ (C, H). Some weak expression of Otx2 was observed in retinal cells located at the vitreal sruface of the central retina (D) which appeared to be negative for CRX expression (C, E). All cells, both RPE and retinal, expressed both CRX and Otx2 at the CMZ (F-J). Scale bars: 20uM (A-E), 50uM (F, G) 100uM (H-J).

5.1.4 Discussion:

In order to have a better appreciation of the expression profile of native human RPE cells, it was necessary to undertake an immunohistochemical analysis of early human fetal eyes. Unfortunately, it was not possible to obtain sections of human fetal eyes at a developmental stage where RPE is first being formed. Similarly, the human tissue available was not necessarily directly comparable with RPE cells in embryonic chick and rat eyes, which are observed to undergo transdifferentiation in response to bFGF treatment. Nevertheless, the developmental stages available were only slightly more developed than those which might expected to undergo transdifferentiation, and therefore still provide a good basis for analysis of the RPE expression profile of immature RPE cells.

5.1.4.1 Bestrophin1:

Bestrophin1 is an RPE specific protein which is known to be crucial for the proper function of the RPE cells, however, it is not clear at what point in RPE development bestrophin1 begins to be expressed (Marmorstein et al., 2000, Esumi et al., 2009). It has been reported to be expressed in stem cell derived RPE cells, for example, HESC-RPE (Vugler et al., 2008) and iPS-RPE (Carr et al., 2009a), which are thought the be representative of an early stage of RPE development. Despite this, the fact that these RPE cells are cultured *in vitro*, and the process of differentiation appears to be variable, means that it is very difficult to accurately developmentally stage the cells with regard to the equivalent *in vivo* counterpart. Characterisation of the expression of different markers in developing human fetal retina will hopefully help to ratify this problem. Bestrophin1 is often regarded as a marker of differentiated RPE cells given its important role in RPE function in vision, therefore, it's expression in heavily pigmented RPE cells of the central developing retina, at the earliest developmental stages available, CS18 and CS23, was perhaps surprising. This expression of

bestrophin1 was observed to be of baso-lateral distribution in the cell membranes of RPE cells, which is consistent with the characteristically membrane-localised, bestrophin1 expression expected (Marmorstein et al., 2000, Esumi et al., 2009). The expression pattern was conserved throughout the whole RPE monolayer. This includes the CMZ region at the periphery of the RPE at all stages of development investigated. At the earliest developmental stage analysed, CS18, some of the cells in the CMZ that were not pigmented, were observed to robustly express bestrophin1. This may suggest that the cells in this region are not fully committed towards the RPE lineage, and therefore do not exhibit pigmentation, but do express some RPE markers. However, given the fact that bestrophin1 expression appears to be regulated by the same transcriptional signalling cascade as proteins involved in the pigmentation of RPE cells, including Otx2 and Mitf (Esumi et al., 2009, Martinez-Morales et al., 2001, Westenskow et al., Mochii et al., 1998a, Shibahara et al., 2000, Tachibana et al., 1996), one would expect cells expressing bestrophin1 to also be pigmented. This may suggest the existence of an additional regulatory pathway for bestrophin1 expression. Despite the apparent lack of intense pigmentation in this bestrophin1 positive, CMZ region, some cells do exhibit small granules of pigment in their cytoplasm. Given the fact that no non-pigmented cells in the CMZ at later developmental stages express bestrophin1, this may suggest that non-pigmented, bestrophin1 positive cells at CS18 are simply in the process of down-regulating bestrophin1, and that it is lost at a different rate to cell pigmentation.

Interestingly, at later stages of development, F2 and F3, bestrophin1 expression was not observed in the heavily pigmented RPE cells of the central retina, unlike RPE at earlier stages. Despite this, bestrophin1 expression was still present in the CMZ region, which suggests that at F2 and F3, bestrophin1 may be down regulated in central RPE cells as a feature of its development, and must therefore be reactivated later in development when required for vision. This observation may suggest a role for bestrophin1 in the maturation/development of RPE cells in the eye, however, if this is the case, the nature of this role remains unclear.

Bestrophin1 has been reported to be important in the early development of a number of ocular structures, including the RPE, which may reflect the restricted expression pattern observed

here (Yardley et al., 2004). Bestrophin1 expression present in the CMZ region of eyes which do not express the protein in more central regions, may indicate the presence of a central to peripheral developmental gradient in the RPE cells at these stages. It would be interesting to analyse whether or not bestrophin1 expression in the CMZ region is eventually down-regulated at later stages, should this tissue become available. If so, then this would support the idea of a central to peripheral developmental gradient. The fact that bestrophin1 expression appears to be variable around the stages of development which are most likely to be of interest with regards to RPE transdifferentiation would perhaps suggest that bestrophin1 is not the best marker of RPE lineage available for future analysis. However, only one biological repeat was available for analysis at each developmental stage, so it is possible that the apparent down-regulation of bestrophin1 in central RPE cells may not be representative of the true expression profile of bestrophin1 at these stages. In order to be sure, analysis of other embryos at these stages would be necessary to confirm the observed spatio-temporal expression patterns.

5.1.4.2 Pmel17:

Another marker that is often used to identify RPE cells is Pmel17 (Vugler et al., 2008, Muller et al., 2007, Carr et al., 2009b). This protein is found in both pigmented RPE cells, as well as cells which are under-going/losing RPE differentiation, including dissociated HESC-RPE cells. Dissociated HESC-RPE cells are observed to lose their characteristic pigmentation and RPE morphology as a result, while retaining the expression of Pmel17 (Vugler et al., 2008). Pmel17 proved to be a good marker of developing RPE cells in embryonic human tissue as all pigmented RPE was labelled for the protein throughout the monolayer, regardless of the developmental stage, or region of analysis. However, at stages CS23 and F1, Pmel17 expression was unexpectedly observed in non-pigmented, neuroepithelial tissue, immediately adjacent to the RPE monolayer. As previously discussed, Pmel17 expression is not normally associated with non-pigmented cells, except for de-differentiated RPE cells, and therefore, to

our knowledge, this is the first report of Pmel17 in native, non-pigmented neuronal cells. These Pmel17 positive cells were confirmed to express immature retinal markers: Chx10, nestin, and Pax6, which implies that they have a neuro-retinal identity. Interestingly, the Pmel17 expression was highest in the neuroepithelium immediately adjacent to the RPE, and its expression was reduced in a gradient towards the middle of the neuroepithelium. This might suggest the involvement of an antagonistic signalling gradient, which is acting to specify the phenotype of the multipotent progenitor cells in the developing optic cup, with proximal cells differentiating into RPE cells, and more proximal cells as neural retinal cells. The expression of both RPE marker Pmel17, and retinal progenitor markers, Chx10, Pax6 and nestin in these cells may imply that these cells are yet to be fully committed the either lineage, with cells adjacent to the RPE experiencing a higher concentration of RPE specifying factors than those in the middle of the neuroepithelium. This would mean that these cells are exposed to more retina-specifying factors. If this is the case, the identity of factors which may be involved remains to be confirmed, however, given the known antagonistic relationship between TGFβ/Activin signalling, which signals from the back of the eye and encourages RPE differentiation (Fuhrmann et al., 2000b, Fuhrmann et al., 2000a, Fuhrmann, 2010), and FGF signalling, which signals from the lens and surface ectoderm, and promotes retinal differentiation (Zhao et al., 2001, Nguyen and Arnheiter, 2000, Hyer et al., 1998, Vogel-Hopker et al., 2000, Galy et al., 2002), it is possible that these pathways may be involved. A similar expression pattern of Pmel17 in the retina has been observed through ectopic application of BMP signalling family members into the developing eye of the embryonic chick (Muller et al., 2007), which may suggest the involvement of BMP signalling factors in the induction of RPE markers in cells of the presumptive retina. However, it is possible that this pattern of Pmel17 expression in the presumptive retina is more reflective of retention of some RPE phenotypic properties, rather than the direct, specific action of BMP factors, which are known to augment the RPE phenotype during development. The same phenotype might therefore be observed with the ectopic addition of other RPE specifying factors. No Pmel17 was observed in the neuroepithelium of eyes at other stages of development investigated,

which would perhaps suggest that the presence of Pmel17 in the presumptive retina is as a result of some specific developmental function at these stages. However, it is worthy of note that these sections of fetal eye tissue did not encompass the optic nerve head, and was instead a more peripheral region of tissue. Therefore, it is possible that retinal cells in this region are subject to a different micro-environmental signalling environment than those adjacent to the optic nerve head, which may involve greater exposure to growth factors emanating from the extra-ocular mesenchyme, known to be important for RPE specification (Fuhrmann et al., 2000b). However, growth factors being released from elsewhere in the eye may also be involved.

In addition to Pmel17 being observed in the presumptive neuroepithelium of the more central regions, Pmel17 was also observed in seemingly non-pigmented cells at the CMZ. This observation was made at all developmental stages analysed. Close inspection of the images does show the presence of a few, very small pigment granules in these regions, which may be responsible for positive Pmel17 labelling, implying that Pmel17 is a very sensitive marker of RPE lineage. However, given the known plasticity of RPE cells, particularly during development, it is possible that the RPE cells in the CMZ are proliferative and transdifferentiating to contribute cells in this region, in order to contribute to the overall growth of the eye, although, the apparently non-neuronal identity of the cells in this region (Chx10, nestin negative) would seem to rule out the presence of RPE to retinal transdifferentiation. In order to confirm this hypothesis, it will be necessary to analyse the same region for proliferative markers, should additional tissue become available. It is interesting to note that these non-pigmented cells of the CMZ have been observed to display a certain plasticity *in vitro*, where they can be cultured to display characteristics of RPE differentiation, including RPE marker expression, and the phagocytosis of latex beads (Vossmerbaeumer et al., 2008), however, this phenomenon may be non-specific since the cells haven't been shown to phagocytose rod outer segments.

5.1.4.3 Pax6:

Pax6 expression was absent from the centrally located, pigmented RPE cells at all stages of development investigated, which was to be expected of post-optic cup stage RPE cells which have continued to mature. This is consistent with a progressive down-regulation of Pax6 in the RPE in animal models of RPE development, such as the chick (Spence et al., 2007b), as well as previous studies in human fetal eyes, which investigated Pax6 expression over a similar developmental window (Nishina et al., 1999, Larsen et al., 2009). This is to be expected given that an up-regulation in Pax6 in the RPE has been associated with an induction of transdifferentiation towards a neuro-retinal phenotype (Azuma et al., 2005a, Spence et al., 2007b), and consequently, down-regulation of Pax6 appears to be important for maintenance of the RPE phenotype. However, contrary to previous reports in human RPE (Nishina et al., 1999), as well as observations made in chick RPE, Pax6 expression was observed to be present in the pigmented CMZ of fetal eyes at all stages investigated. It is possible that a previous report did not detect this expression of Pax6, owing to the fact that they did not utilise fluorescent labelling. It is reasonable to assume that any positive signal would therefore be obscured by the heavy, RPE pigment present within these cells. The reason for this Pax6 expression was unclear, however, it could suggest that RPE cells in more peripheral regions of the eye retain a level of plasticity that is lost in more central regions, given the apparent association of Pax6 expression, with the retention of the capacity for transdifferentiation. The most obvious explanation for the observed proximo-distal increase in expression of Pax6 in the RPE, would be the presence of a proximo-distal gradient in the developmental age of RPE cells, given that centrally located RPE are generally considered more mature than their peripheral counterparts. However, the fact that this gradient does not appear to change with a progression of development would suggest that this is not the case. Instead, the proximo-distal gradient may reflect the proximo-distal gradient in FGF signalling. This is present as a result of FGF's being released from the surface ectoderm (Nguyen and

Arnheiter, 2000, Hyer et al., 1998), with antagonistic TGFβ-like proteins being released from the extra-ocular mesenchyme at the back of the eye (Fuhrmann et al., 2000b). Given the link between FGF signalling and the up-regulation and maintenance of Pax6 expression (Spence et al., 2007b, Kuriyama et al., 2009a), it is possible that this could be the reason why Pax6 is retained in the CMZ. It appears that Pax6 expression must reach a threshold level in order to undergo transdifferentiation, given the Pax6 expression within immature RPE cells (Spence et al., 2007b), as well as HESC-RPE monolayers in culture (Vugler et al., 2008, Klimanskaya et al., 2004) that both retain their characteristic phenotype. The reduced expression of Pax6 in RPE cells compared to the adjacent neural retina may suggest that any threshold for transdifferentiation has not been met. This would explain why the cells have retained the RPE phenotype. If FGF signalling is responsible for the Pax6 expression in the RPE, this has yet to be confirmed. Despite this, if the identity of the signalling pathways responsible for the maintenance of Pax6 in RPE cells can be elucidated, this may allow the expression of Pax6 in RPE to be modulated, and therefore potentially regulate the phenotype of the RPE, including its capacity for transdifferentiation.

The presence of Pax6 expression in these cells may also suggest that they are not in fact RPE cells, but iris pigmented epithelium (IPE), which is known to express Pax6 when isolated from adult patients (Froen et al., 2011), and is likely to be located in a similar region to Pax6 positive cells observed in early development. However, it was reported that these cells were also known to express nestin, which was absent from these cells at this stage. Similarly, IPE are also known to express Sox2, however, more tissue will be required in order to analyse the expression of other markers. It is possible that nestin expression becomes initiated in the Pax6 positive cells later in development. Maintenance of Pax6 expression in IPE cells appears to be important for the formation of the lens, in addition to transdifferentiation towards a lentoid phenotype as a response to various treatments (Asami et al., 2007, Kosaka et al., 1998).

5.1.4.4 Chx10 & nestin:

Chx10 and nestin expression was present throughout the neuroepithelium at all stages investigated, which is consistent with its identity as a presumptive retina containing retinal progenitors (Ahmad et al., 1999, Vossmerbaeumer et al., 2008) (stage F1) (Chen and Cepko, 2000, Fuhrmann et al., 2000b, Horsford et al., 2005, Belecky-Adams et al., 1997). No Chx10 or nestin was present in the CMZ, which suggests that this region does not have a neuro-retinal identity *in vivo*, however, there is some evidence to suggest that culturing of these regions *in vitro* can lead to an up-regulation in neural progenitor markers like nestin (Vossmerbaeumer et al., 2008, Ahmad et al., 2000). It remains unclear what structures of the eye these cells will go on to develop, however, it is possible that this region, in addition to Pax6 positive pigment epithelial cells, go on to form the ciliary body.

5.1.4.5 Otx2 & CRX:

Otx2 has been heavily implicated in the development of both RPE cells and retinal cells (Nishida et al., 2003, Sakami et al., 2005, Viczian et al., 2003, Akagi et al., 2004, Martinez-Morales et al., 2003, Martinez-Morales et al., 2001, Martinez-Morales et al., 2004, Zuber et al., 2003, Bovolenta et al., 1997, Bobola et al., 1999, Koike et al., 2007, Esumi et al., 2009, Larsen et al., 2009, Takeda et al., 2003, Glubrecht et al., 2009, Beby et al., 2010). As expected from previous reports (Larsen et al., 2009), all RPE cells were observed to express the transcription factor within their nucleus, however, perhaps more surprisingly, this expression co-localised with the photoreceptor associated transcription factor, CRX. CRX expression had been previously observed to co-localise with Otx2 expression in cultured embryonic chick RPE cells, and has been reported to be expressed in bovine RPE (Esumi et al., 2009), as well as both cultured and native human RPE cells (personal communication,

unpublished data)(Glubrecht et al., 2009). Given the high level of sequence identity between Otx2 and CRX, it is possible that there is some level of cross-reaction between the antibodies, which may recognise epitopes on both proteins. However, not all cells that were labelled for Otx2 in the retina were also labelled for CRX, which may indicate that the CRX signal within the RPE is indeed real. It is unclear what the function CRX expression in the RPE would provide, however, it has been reported that CRX is able to bind the promoter region of RPE specific gene, BEST1, which encodes RPE functional protein, bestrophin1, and increase its expression, in a similar manner to Otx2 (Esumi et al., 2009). Therefore, it could be that CRX is responsible for the initiation of transcription for a number of genes that encode RPE machinery. Otx2 in the retina is largely confined to the cells that are immediately adjacent to the RPE monolayer in F1 tissue. This is consistent with a previous report in human tissue at a similar stage (Larsen et al., 2009), and suggests that Otx2 expression at this stage is largely confined to the developing, CRX positive, photoreceptor layer (Glubrecht et al., 2009), as is characteristic of a more mature retina (Rath et al., 2007, Koike et al., 2007). This is consistent with its role in the transactivation of both IRBP (Bobola et al., 1999) and CRX expression in photoreceptor cells (Nishida et al., 2003), and co-transfection of both Otx2 and CRX into adult iris/ciliary tissue is reported to induce photoreceptor characteristics in these cells (Akagi et al., 2004). Some Otx2 positive cells which are found towards to middle of the retinal neuroepithelium may also be differentiating into bipolar cells given the implication of both transcription factors in bipolar cell development (Bovolenta et al., 1997). Interestingly, despite usually being associated with RPE, photoreceptor, and bipolar cell fates, Otx2 (CRX negative) positive cells observed in the retina have been identified as post-mitotic neuroblasts, which are appear to be under-going differentiation into a number, if not all of the different retinal cell types, as displayed through its co-expression with several specific retinal cell markers (Bovolenta et al., 1997). The apparent ability of Otx2 to transactivate the expression of retinal cell specific markers such as CRX may imply that it can also initiate the expression of other key transcription factors involved in facilitating retinal cell differentiation. Once

again, it would be very interesting to analyse the expression of both Otx2 and CRX at a number of other developmental stages, potentially in conjunction with other retina specific markers, in order to unravel their function in development in the future.

5.2 What is the effect of bFGF treatment on the phenotype of human fetal RPE CS21 explants after 10 days in culture? Is transdifferentiation initiated?

5.2.1 Introduction:

Despite the fact that a number of different organisms have been shown to undergo the phenomenon of RPE to neuroretinal transdifferentiation, it remains unclear whether or not human RPE cells can also undertake this process in a similar manner to that of animal models.

A sub-population of adult human RPE cells has been reported to display multi-potent characteristics using defined treatments *in vitro*, which are reported to produce new cells with both mesenchymal and neural phenotypes (Salero et al., 2012). Additionally, it is already known that immortalized, human RPE cell lines (such as ARPE19) are able to undergo a limited phenotypic change in culture, in response to treatment with retinoic acid analogue, fenretinide (Carr et al., Chen et al., 2003, Chen et al., 2006). This involves a change from a distinct epithelial morphology, towards a typical neuronal morphology with cells exhibiting extended neuron-like processes. Accompanying this morphological change are expression of a number of neural and retinal markers not usually associated with RPE cells, including: paired-box 6 protein (Pax6), cone-rod homeobox protein (Crx), sex-determining Y-box 2 (Sox2) and neural retina leucine zipper (Nrl). Interestingly, many of the markers indicating a retinal progenitor state are expressed in both the fenretinide-treated RPE cells as well as DMSO-treated controls. This would indicate that, under standard culture conditions, these types of RPE cells are pre-disposed to de-differentiate away from a typical RPE phenotype, and move towards a neural progenitor state. This is also evident in the fact that under standard culture conditions, ARPE19 cells contain little pigment, if any at all – an attribute which is

strongly associated with de-differentiating RPE cells (Liu et al., 2009b). Despite the fact that fenretinide treatment has been reported to yield a decrease in RPE markers, such as Otx2 (Simeone et al., 1995, Carr et al., Chen et al., 2006, Chen et al., 2003), and an increase in the expression of retinal markers, such as cone, long-wave sensitive opsin (OPN1lw), rhodopsin expression has not been reported (Carr et al.). Additionally, some of the markers which would normally be associated with the neural retina are also found to be expressed in ARPE19 cells prior to treatment with fenretinide (Carr et al.). Therefore, it remains unclear whether this method of inducing transdifferentiation would be effective on RPE cells which do not already express retinal progenitor markers, having already apparently undergone de-differentiation, and also whether or not it could produce the functional rod photoreceptors required for transplantation. It is possible that the lack of rhodopsin expression in these cultures was due to the use of a two-dimensional culture system, given that retinal development is known to rely heavily on the complex, three-dimensional, expression pattern of various factors during development (Fuhrmann, 2010). These signaling mechanisms would most-likely be heavily disrupted in a two-dimensional culture system; resulting in improperly developed retinal cells.

Given the need for properly developed, functional retinal cells for transplantation, it may therefore be more appropriate to try and replicate the type of 'classical transdifferentiation' more commonly observed in animal models such as amphibians, embryonic chickens and embryonic rats. This type of transdifferentiation utilizes a three-dimensional culture system, akin to that of the developing mammalian retina. As a result, it is reasonable to assume that retinal cells are more likely to develop properly given that they are more likely to receive the correct micro-environmental cues resulting from an intact retina. There are relatively well-established protocols for the induction of transdifferentiation in these organisms that should easily translate into use with human-derived RPE cells (as demonstrated in chapters 3 & 4). RPE explants are cultured in a non-adherent culture system and treated with basic fibroblast growth factor (bFGF/FGF2) for a number of days. RPE cells are observed to lose their pigmented, epithelial phenotype, and form large loops of low optical density, neuroepithelium, which protrude from the floating spheres of RPE explants

(Pittack et al., 1997, Sakaguchi et al., 1997, Zhao et al., 1995, Sakami et al., 2008, Reh et al., 1991, Kodama and Eguchi, 1995, Pittack et al., 1991). These neuroepithelia appear to exhibit a structure and cellular morphology similar to that of the developing neural retina. In addition, markers of a number of different retinal cell types are reported to be expressed in a spatial pattern typical of a developing retina, including rhodopsin expression in rats, which implies the presence of rod photoreceptors (Zhao et al., 1995). Crucially, this type of transdifferentiation only occurs when intact monolayer, sheets of RPE are treated with bFGF, indicating that the physical configuration of cells in important for proper transdifferentiation (Reh et al., 1991). Dissociated cells do not display the same capacity for transdifferentiation towards developed retinal cells, if any at all (Pittack et al., 1991). Similarly, RPE cells cultured in contact with a surface have been shown to display variable ability for transdifferentiation (Opas and Dziak, 1994b). The capacity for transdifferentiation of the RPE is reported to be dependent on the mechanical properties of the substratum itself, as well as its composition (Opas and Dziak, 1994b, Reh et al., 1987). It remains unclear why RPE cells only undergo full transdifferentiation when cultured as intact sheets, however, it may be a result of the fact that dissociation of RPE cells is known to induce de-differentiation of the cells towards a mesenchymal phenotype (Zhao et al., 2001). This may affect the ability of the RPE cells to transdifferentiate towards retinal cells of neuroectodermal origin. Additionally, non-adherent culture may promote the response of RPE to extracellular signals, like bFGF, as well as providing the correct mechanical properties to allow the migration of transdifferentiated RPE cells to produce a new, neuroepithelial structure. Indeed it has been shown that *in vitro* transdifferentiation of *xenopus* RPE is able to regenerate all retinal layers *in vitro* when cultured in the presence of overlaid extracellular matrices, which allow the RPE cells to migrate and transdifferentiate (Kuriyama et al., 2009b).

Given the above, although immortalized human RPE cell lines, such as ARPE19, have shown some capacity to undergo transdifferentiation, they are perhaps not the best model for investigation of 'classical transdifferentiation,' for a number of different reasons. These types of RPE cell line have been shown to exhibit an unconventional gene expression

profile, with many expressing unexpected markers of retinal cells (Carr et al., Chen et al., 2006). These markers are not usually associated with differentiated RPE cells. In addition, they don't necessarily exhibit the classical, cobblestone morphology and intense pigmentation usually associated with RPE cells. Practically speaking, it may be difficult to obtain intact sheets of cells from these cell lines, as they do not appear to have as strong integrity as native RPE. It is also unclear, given that these cell lines have undergone multiple passages (and therefore dissociations), whether this may have affected their ability to undergo classical transdifferentiation. Particularly given that other mammalian models of RPE transdifferentiation, including the rat and the mouse, only display this ability at a very early, restricted stage of development (Coulombre, 1981, Coulombre and Coulombre, 1965, Pittack et al., 1997, Pittack et al., 1991, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993, Sakami et al., 2008, Reh et al., 1991). The likely-hood of developmental age also being a factor for transdifferentiation of human cells is high, given that the phenomenon appears to be conserved in a number of species, including the chicken and rat (Zhao et al., 1995). This is in contrast to the newt, which appears to have unique properties of regeneration (Avdonin et al., 2008, Chiba et al., 2006b, Eguchi, 1988, Ikegami et al., 2002, Kaneko and Chiba, 2008, Kuriyama et al., 2009a, Mitsuda et al., 2005, Sakami et al., 2005, Susaki and Chiba, 2007). There is therefore a concern surrounding the use of human RPE cell lines that have been expanded in culture for a large amount of time, as this is likely to have affected their capacity for transdifferentiation. Similarly, if developmental age is important for transdifferentiation, it would be potentially very difficult to accurately 'age' RPE cells in culture when compared with other sources of human RPE cells. Especially given that RPE cells in culture may develop in a different manner to those *in vivo.*

Therefore, because of these potential issues using established human RPE cell lines, it is preferable to use primary human RPE cells to initially investigate the potential for human RPE transdifferentiation. Despite the supply of this type of tissue being limited, it is arguably a more apt model for studying transdifferentiation because these cells, by definition, have the standard RPE cell expression profile, cobblestone morphology, and pigmentation. It is also

relatively easy to separate primary RPE cells from other surrounding tissues, in order to obtain intact sheets of RPE (as with animal RPE explants). Crucially, it is also possible to obtain early embryonic material, which one might reasonably expect to have a greater capacity for transdifferentiation than later stage RPE tissue, if human tissue behaves like its animal counterparts. It is also possible to accurately determine the developmental stage of this tissue, which is important for investigating the potential limitations for induction of transdifferentiation in RPE cells.

In order to investigate whether or not human RPE cells have the capacity for transdifferentiation, human fetal RPE at Carnegie stage 21 (52 days/approximately 7 weeks), the earliest available human RPE tissue at the time of investigation, was treated with bFGF in standard, non-adherent culture system, known to induce transdifferentiation in animal models.

5.2.2 Materials & Methods:

5.2.2.1 RPE cell culture:

Human fetal RPE tissue at CS21 was dissected and cultured in a standard, non-adherent, transdifferentiation, culture system +/- bFGF (100ng/ml) for 10 days as discussed in chapter 2.2/2.3.

5.2.2.2 Immunohistochemistry:

Immunohistochemical, and statistical analysis was performed as described in chapter 2.4.

5.2.2.3 Image analysis:

In order to quantify the level of expression of different proteins in both bFGF treated, and untreated human fetal explants, it was necessary to employ the use of image analysis software, ImageJ, to measure the average pixel intensity in the confocal images. The average pixel intensity for the overall section of low magnification images was quantified as described in Fig.. A number of low magnification images were converted to 16-bit grayscale images (Black = 0, White = 255), which were then cropped using the *region of interest* tool in order to select the explanted RPE area of each section to be analysed. The cropped region was then analysed using ImageJ in order to quantify the average pixel intensity within the whole section. The fact that only n=1 biological repeats were available meant that it was necessary to take several measurements of both Pmel17 and pigmentation levels, from several different sections of the same explants (+/-bFGF), in order to gain an appreciation of the level of error in the values. It was possible to do this for Pmel17 and the level of pigmentation as the nomarsky channel was present for all images, regardless of which flurorescent markers were being investigated, and additionally, a number of different sections were also labeled for Pmel17 in conjunction with other markers, given its identity as a robust RPE cell/phenotype marker.

For other markers, given the lack of tissue, only one section was available to analyse to expression of each marker, and therefore, it was difficult to ascertain the level of variability/error in the values. It was therefore necessary to apply regional analysis of the average pixel intensity to the available images, rather than whole section analysis as above. The method by which regional average pixel values were obtained is described in Fig. 5.M2. Images were converted to 16-bit grayscale images as before, however, in this instance, only small regions of approximately equivalent size were selected for analysis, using the *region of interest* tool. These values were subsequently averaged and the repeated values were used to calculate the standard error of the dataset for each marker, including Pmel17 and pigmentation, in each of the two culture conditions (+/-bFGF). Additionally, these regions of interest were used to analyse the corresponding level of pigmentation and expression level of

Pmel17, in exactly the same regions of different channels of the same image, in order to analyse the correlation between different markers.

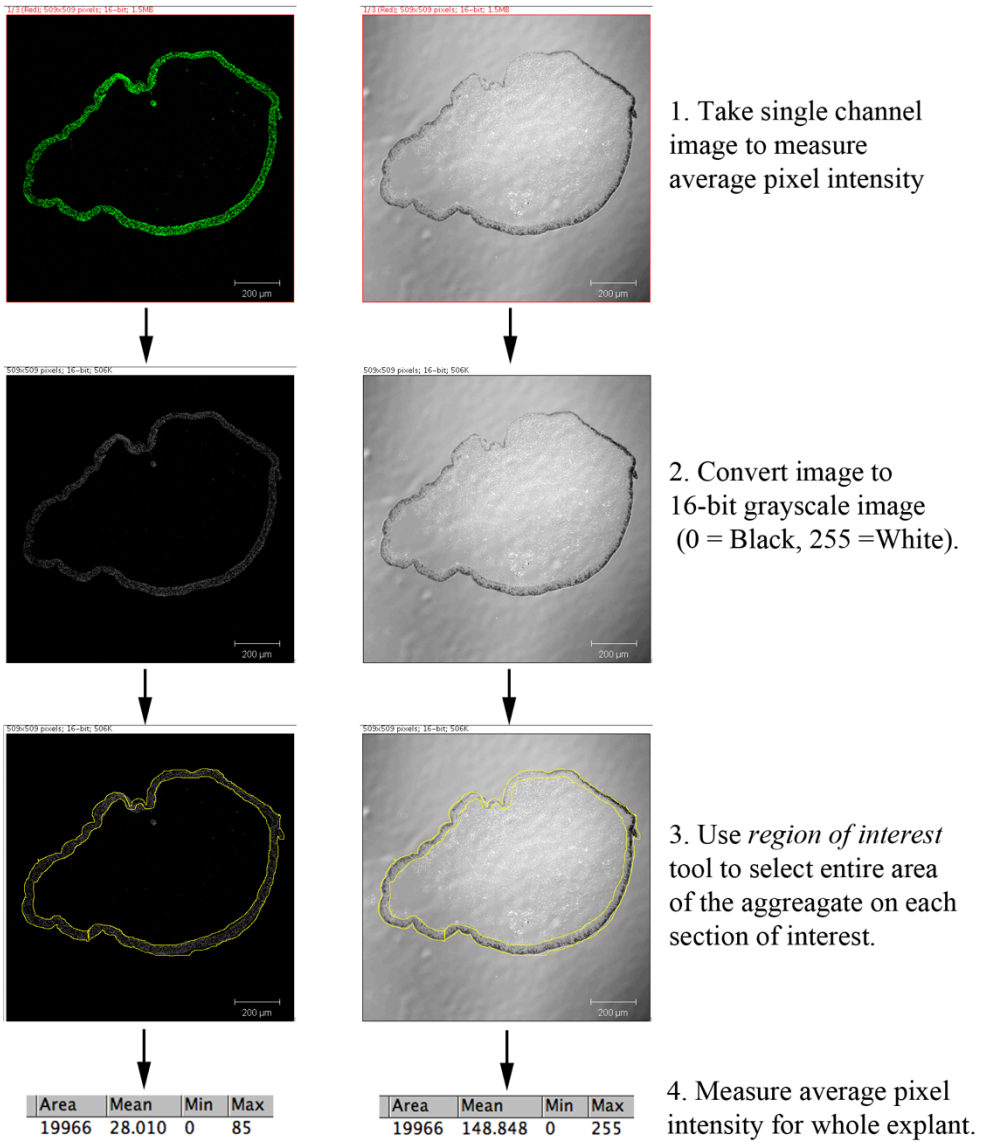
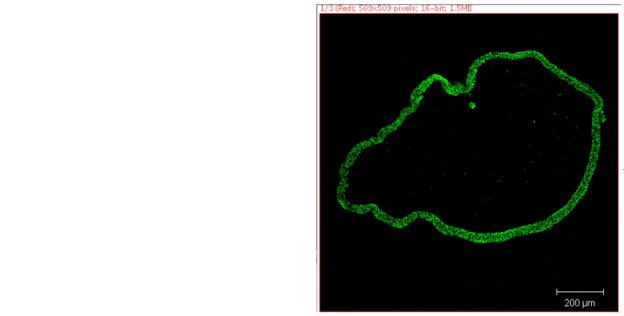
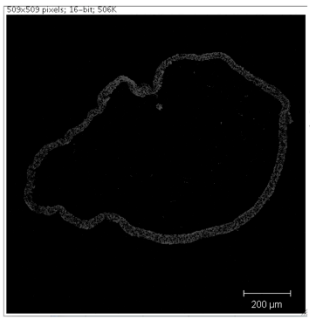


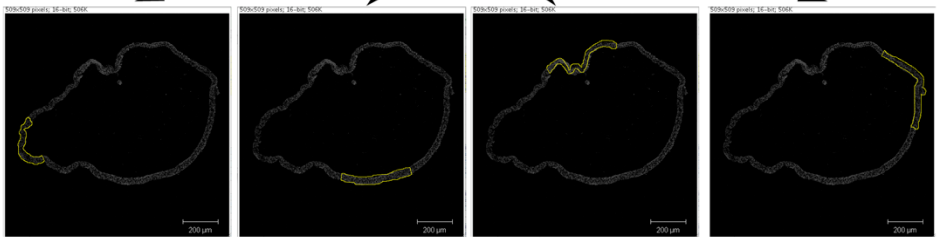
Fig. 5.M1
Whole image analysis of gene expression / level of pigmentation in each section of human fetal RPE CS21 after 10 days in culture. This is the method by which whole sections are analysed for the average pixel intensity of fluorescence/pigmentation. Images are converted into 16-bit grayscale images, and subsequently, whole explants are selected using the region of interest tool. The average pixel intensity can then be measured for each section of the explant.



1. Single channel image to measure in ImageJ.



2. Convert image to 16-bit grayscale image (0 = black, 255 = white).



3. Use *region of interest* tool to crop whole section for average pixel measurement.

Measure

	Area	Mean	Min	Max
1	2010	21.945	0	85
2	2832	34.087	0	85
3	2451	31.477	0	85
4	3345	25.896	0	85

4. Measure average pixel intensity of each region for each channel.

Fig, 5.M2

Quantification of regional average pixel intensity in both flurorescent images and nomarsky images in order to quantify the level of expression of different markers. Images are converted to 16-bit grayscale images and approxiamtely equal areas of 4 regions of interest are analysed for average pixel intensity in each channel of each section. These values were subsequently averaged for each marker. Regions of interest remained the same across different channels of the same section in order to allow the analysis of potential correlation between different markers.

The thickness of the RPE tissue in the explanted aggregates was measured at several points in multiple sections of the cultured explants in order to ascertain whether or not there was a difference between explants cultured with and without bFGF. Four measurements were made for each section taking into account the whole range of thicknesses throughout the entire section. These were then averaged in order to compare the differences between explants cultured +/-bFGF.

5.2.2.4 Statistical analysis

As described in chapter 2.11.

5.2.3 Results:

Intact human fetal RPE CS21 explants did exhibit a number a changes following the treatment with bFGF for 10 days when compared with untreated, negative controls. Both bFGF treated and untreated controls were observed to form floating, spherical aggregates within 24 hours of the start of culture, which retained a characteristic, intensely pigmented phenotype, throughout the time in culture (Fig. 5.7, 5.11, 5.13, 5.14, 5.16, 5.19). The aggregates did not exhibit neuroepithelial protrusions often associated with transdifferentiation of RPE cells towards a retinal phenotype using a similar culture system (Fig. 5.7). Sectioning of the material revealed that the pigmentation was largely confined to the apical surface of cells in the aggregates, which also retained a characteristic epithelial morphology (Figs. 5.11, 5.13, 5.14, 5.16, 5.19). This correlated with an expected expression of characteristic RPE marker Pmel17 (also known as matrix melanosomal protein-115 (MMP115) (Figs. 15.3, 5.14, 5.16, 5.19). It wasn't clear whether or not the level of pigmentation of explant was affected by the treatment with bFGF, and therefore, quantification of the relative pigmentation levels, in addition to the expression levels of Pmel17 in either condition, were undertaken using image analysis software. Overall

expression of Pmel17 in low magnification images of sectioned aggregates was observed to be slightly higher in bFGF-treated explants when compared with untreated controls, however, this effect was not observed to be significant (n=3) (Fig. 5.8A).

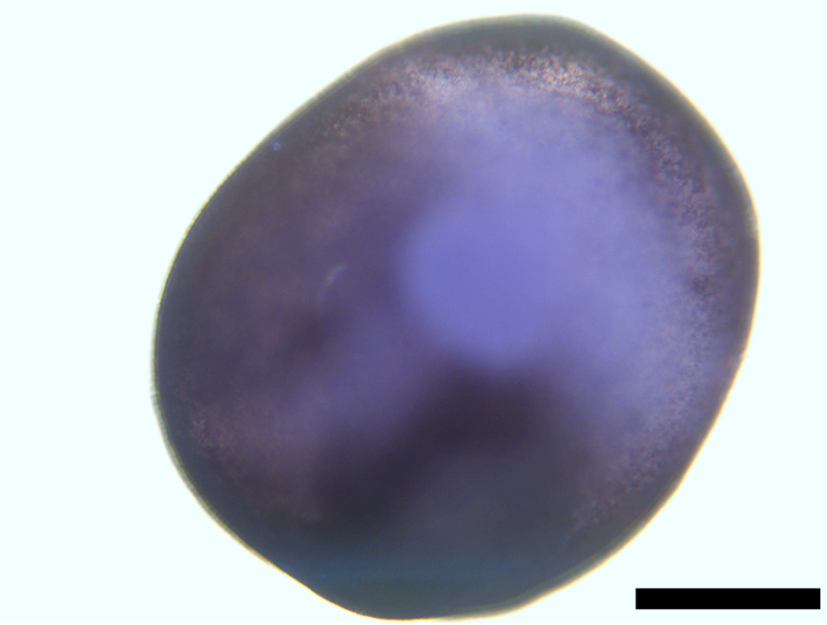


Fig. 5.7
Human Fetal RPE 8 Weeks cultured in non-adherent conditions for 10 days forms floating spherical structure which retains pigmentation.
Despite having been removed from the human fetal eye, the RPE is observed to retain what appears to be a classical, RPE pheontype. This includes heavy pigmentation, in addition to a cobblestone-like morphology. However, instead of remaining as a flat sheet of cells, the RPE monolayer is observed to rapidly form smooth, floating spheres via an unidentified process. Scale bar: 1mm.

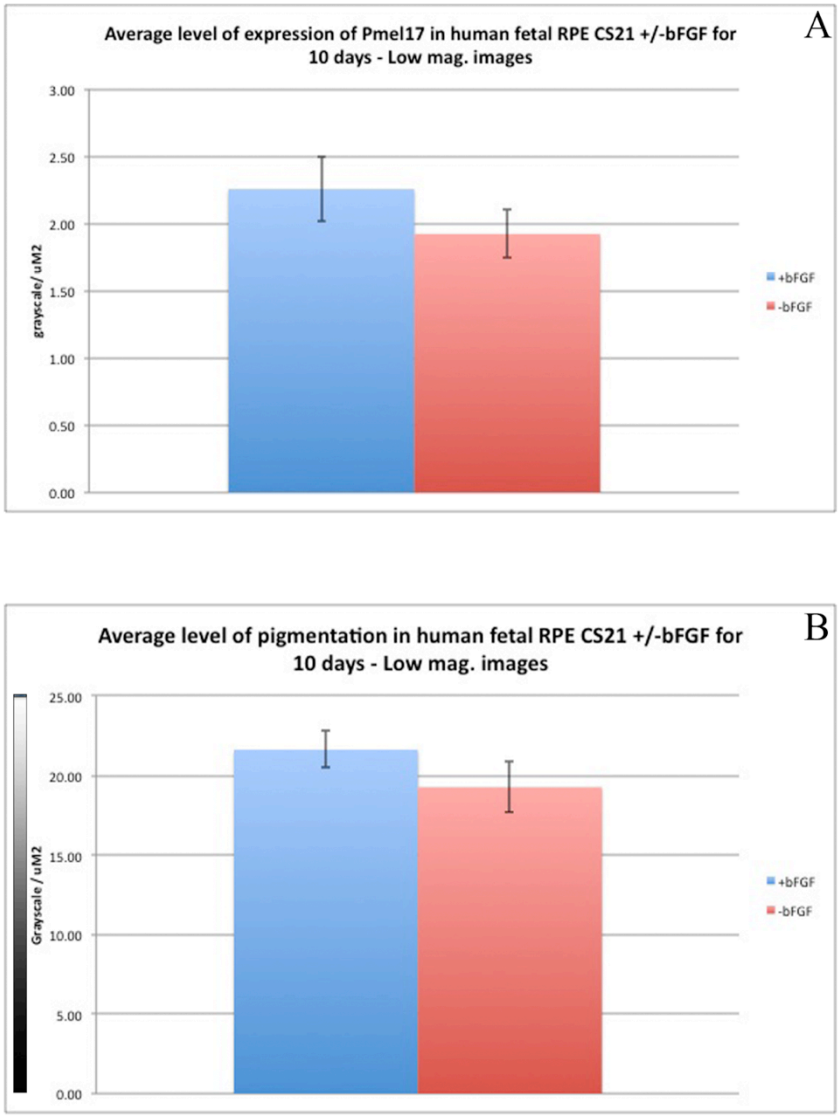


Fig. 5.8
Average expression (pixel intensity) of Pmel17 (A), and level of pigmentation (B) in human fetal RPE CS21 +/-bFGF (100ng/ml) for 10 days. Values averaged from several (n=4) low magnification images (different sections) of the RPE explant (n=1) for each culture condition. No significant difference in either pigmentation (B) or Pmel17 (A) expression between +/-bFGF conditions. This would indicate a lack of effect of bFGF on the maintenance of the RPE phenotype. Error bars : standard error. grayscale: 0-black, 255-white.

The level of pigmentation was measured to be slightly lighter in bFGF-treated explants in comparison to untreated the untreated control, however, once again this difference was not significant (n=3) (Fig. 5.8B). Similarly, analysis of high magnification images of the same explants showed that although average Pmel17 expression was found to be higher in explants treated with bFGF, again this difference was not significant (n=3) (Fig. 5.9A). Surprisingly, high magnification nomarsky images displayed less pigmentation in bFGF-treated explants, compared with untreated explants (Fig. 5.9B), in contrast with lower magnification images (Fig. 5.8B). This difference in the level of pigmentation was also observed to be statistically significant ($p<0.05$, n=3, RANOVA). The level of expression of Pmel17 in different regions of the sectioned explant (low magnification images) did not appear to correlate with the level of pigmentation when a number of different regions of the explant were analysed for their level of pigmentation, and corresponding Pmel17 expression (Fig. 5.10A). A scatterplot of these data show a clustering of datapoints in relatively the same area, with no apparent pattern (Fig. 5.10A). Trendlines for both conditions were relatively horizontal with very low R^2 values: $R^2 = 0.013$ and $R^2 = 0.12$ for –bFGF and +bFGF conditions respectively (Fig. 5.10A). Both bFGF treated and non-treated explant datapoints were clustered in the same region, which once again suggests that bFGF did not effect the expression of Pmel17 or the level of pigmentation in cultured explants, as discussed previously (Fig. 5.8). In order to confirm this, histograms of the average expression levels of Pmel17 (Fig. 5.10C), in addition to the level of pigmentation (Fig. 5.10B) in each *region of interest* analysed for quantification, were plotted. As expected given previous analysis of whole images (Fig. 5.8), on average, explants treated with bFGF displayed lighter pigmentation than those with no exogenous bFGF, however, this difference was not observed to be significant (n=12) (Fig. 5.10B). Similarly, explants treated with bFGF also exhibited slightly increased levels of expression of RPE marker Pmel17 in comparison to untreated controls, however, once again this difference was not found to be significant.

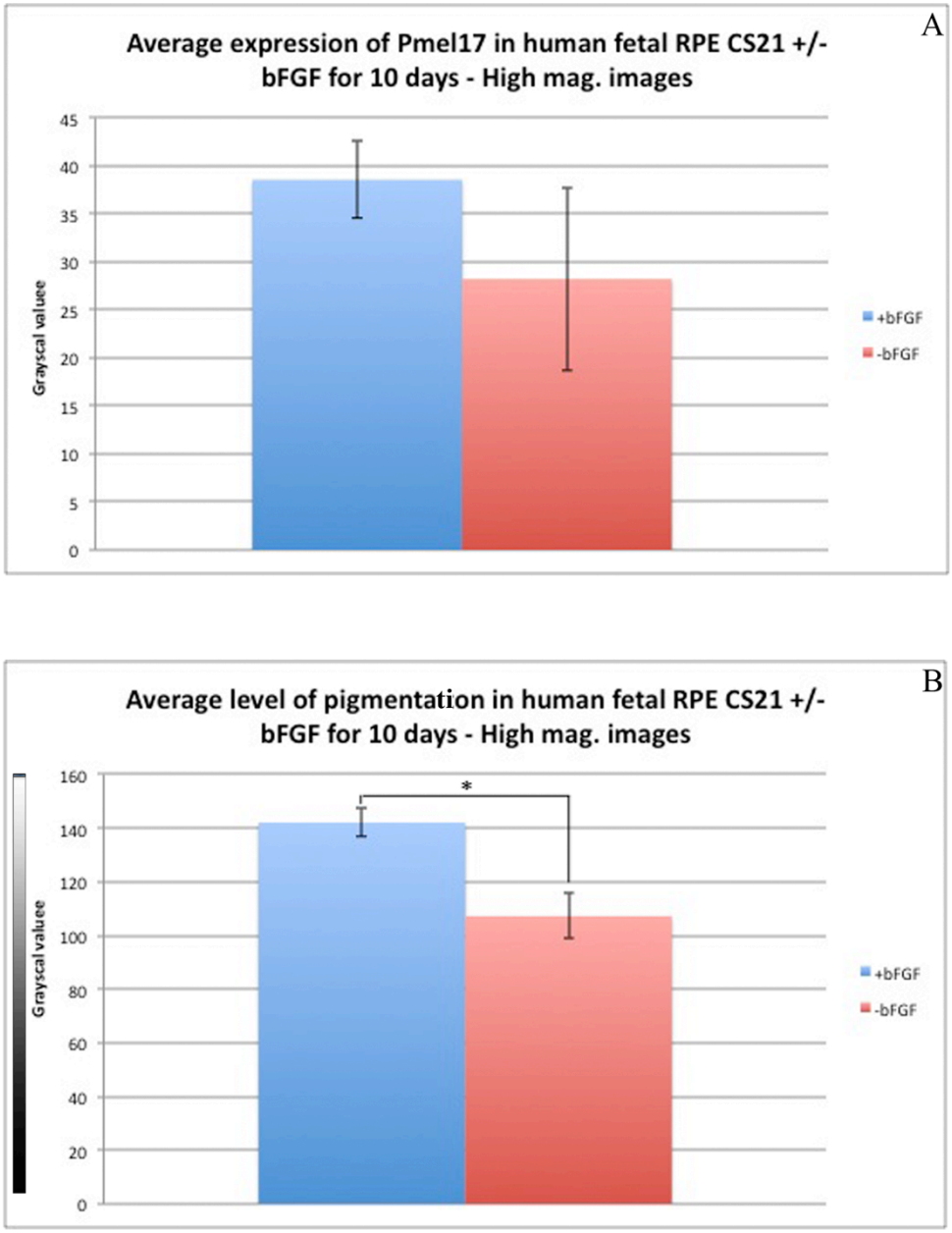


Fig. 5.9
Average expression (pixel intensity) of Pmel17 (A), and level of pigmentation (B) in human fetal RPE CS21 +/-bFGF (100ng/ml) for 10 days. Values averaged from several high magnification images (different sections) of the explant (n=1) for each culture condition. No significant difference in Pmel17 expression was observed between +/-bFGF culture conditions (A), however, explants cultured with bFGF were significantly less pigmented than untreated control (B). This is consistent with the idea that bFGF can cause de-pigmentation in the RPE ($p<0.05$, n=4, RANOVA) Error bars : standard error. grayscale: 0-black, 255-white.

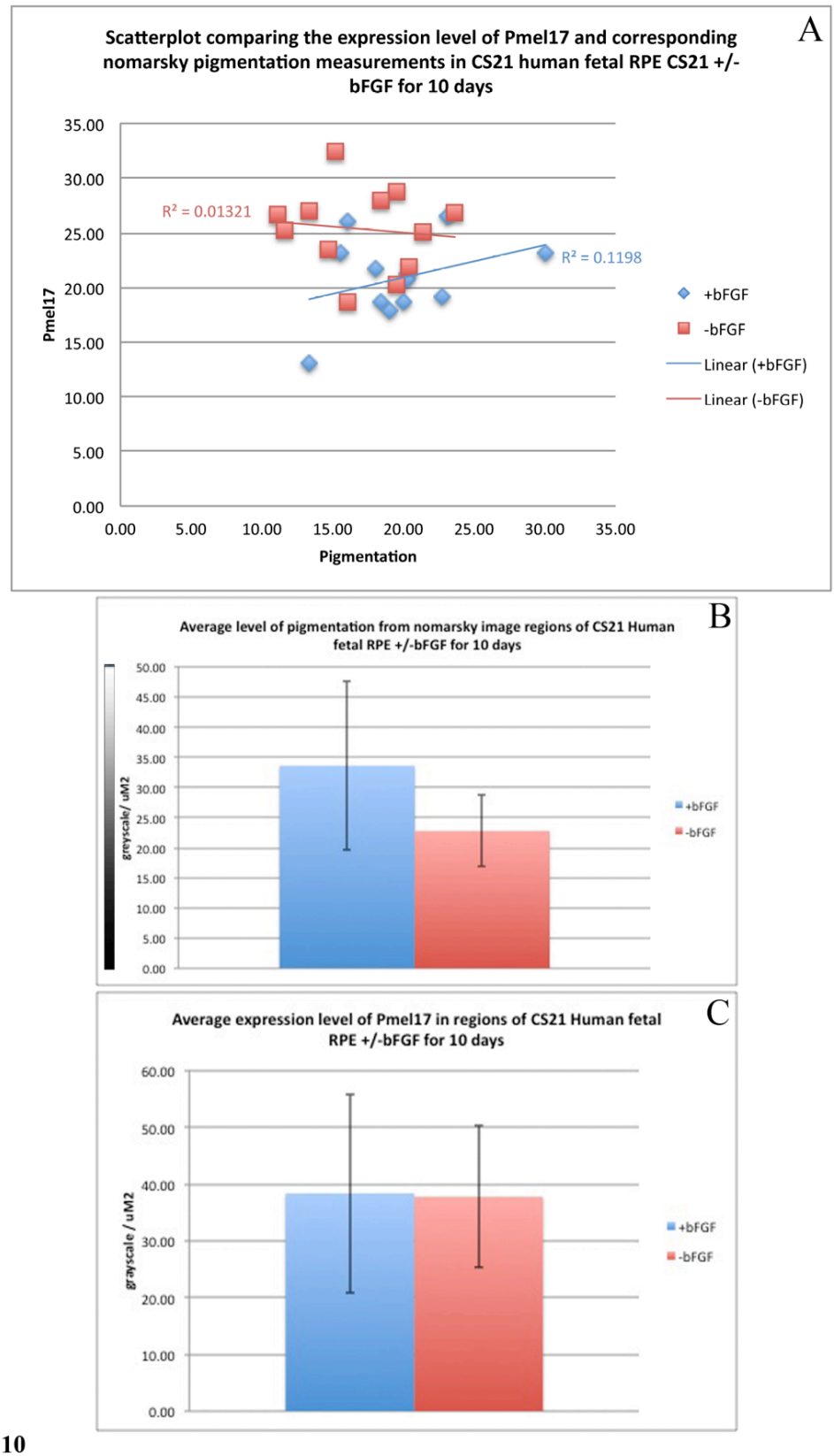


Fig. 5.10

Human fetal RPE CS21 +/-bFGF (100ng/ml) after 10 days. (A) Comparison of the expression level of Pmel17, with the corresponding level of pigmentation, in regions of interest (n=12) of low magnification images. +bFGF (blue) and -bFGF (red) cluster in the same region and have little correlation, as demonstrated by the low Rsquared values for both culture conditions. (B) Histogram showing the average level of pigmentation in the regions of interest of explants cultured in +/-bFGF. No significant difference between treated and untreated explants. (C) Histogram showing the average level of expression of Pmel17 in these regions of interest showing no significant difference in expression between each culture condition. This would suggest that bFGF does not effect the classical pigmented phenotype of the RPE. Error bars: Standard error

Transcription factors Mitf and Pax6 have been heavily implicated in the control of the RPE fate, in addition to the mechanism of RPE to neural retina transdifferentiation. Human fetal explants stage CS21 we observed to express Mitf in the nuclei of all the cells in explants treated both with (Fig. 5.11D), and without (Fig. 5.11C) exogenous bFGF. A cell count of the number of cells expressing Mitf in either condition confirmed that all cells within the aggregates exhibited robust expression of Mitf regardless of growth factor treatment (Fig. 5.12B). The explant treated with bFGF appeared to have a much more disorganized structure of cells (Fig. 5.11B) than that of untreated cells (Fig. 5.11A), with some apparently multi-layered regions within the layer, as demonstrated by the pattern of nuclear staining using DAPI. Pigmentation in the bFGF treated explant appeared to be more localized to the apical surface of the aggregate (Fig. 5.11B) in comparison with that of untreated explants, which exhibited intense pigmentation throughout the cytoplasm of the cells (Fig. 5.11A). bFGF-treated explants appeared to have slightly lighter pigmentation than untreated counterparts in these high magnification images (Fig. 5.11) as previously discussed (Fig. 5.9B). Mitf expression was comparable in both +bFGF and –bFGF culture conditions which suggests that bFGF does not effect the expression level of Mitf in human fetal RPE, at stage CS21 (n=1) (Fig. 5.12A). Both treated and untreated explants were negative for Pax6 expression (Fig. 5.11F and E respectively) in all cells of either explant. Another transcription factor which has been implicated in retinal development, and therefore a switch from an RPE phenotype towards a retinal phenotype, is Chx10. Expression of this transcription factor was not observed in explants cultured with or without bFGF (Fig. 5.13E, F), even in regions of RPE in the +bFGF explant, which were apparently multi-layered as evidenced by the DAPI labeling in comparison to untreated controls (Fig. 5.13A, B). Despite appearing different in morphology to untreated explants, these regions of apparently over-lapping cells retained the robust expression of RPE marker Pmel17 (Fig. 5.13D), in a granular pattern, as in the negative control (Fig. 5.13D). Explants in both conditions retained their pigmentation, however, the explant treated with bFGF appeared to exhibit slightly lighter pigmentation than the –bFGF control.

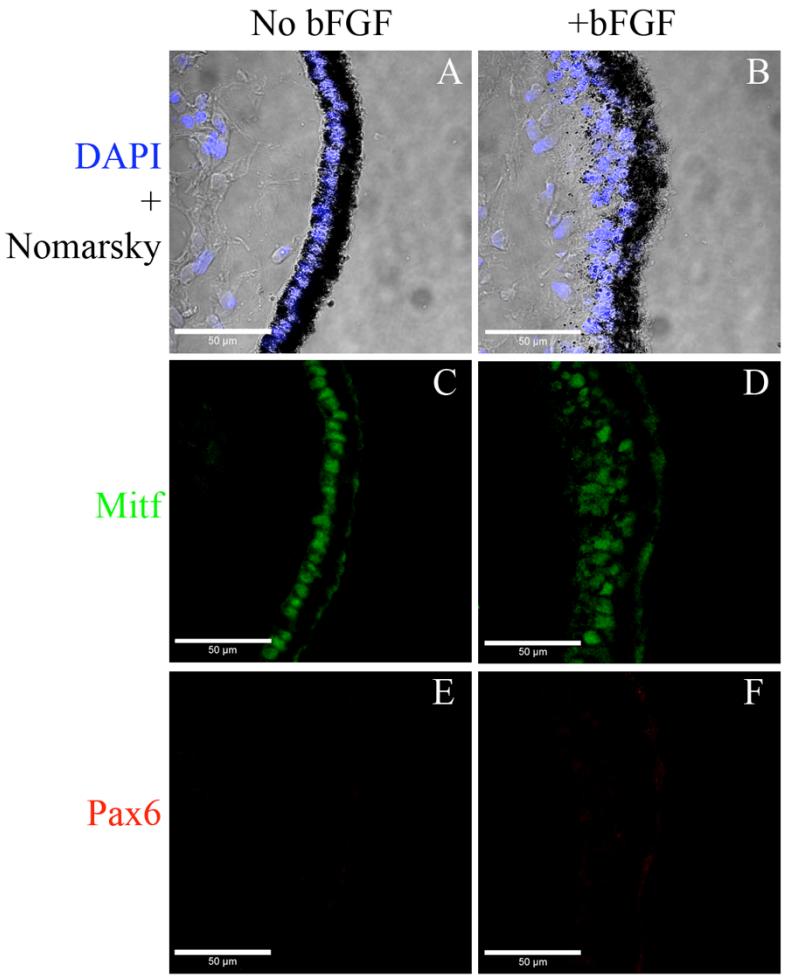


Fig. 5.11
Expression of Mitf + Pax6 in human Fetal RPE stage CS21 treated with bFGF (100ng/ml) for 10 days.
-bFGF (A, C, E), +bFGF (B, D, F). Explants both +/-bFGF retained their pigmentation (A, B), however, the explant +bFGF appeared less epithelial than the negative control, with some apparently multi-layered cells (DAPI, blue, B). Expression of Mitf (green) is found in all nuclei of explants cultured both with and without bFGF (C, D). Neither explants treated with bFGF (F) or without bFGF (E) were observed to express Pax6. Scale bars: 50uM.

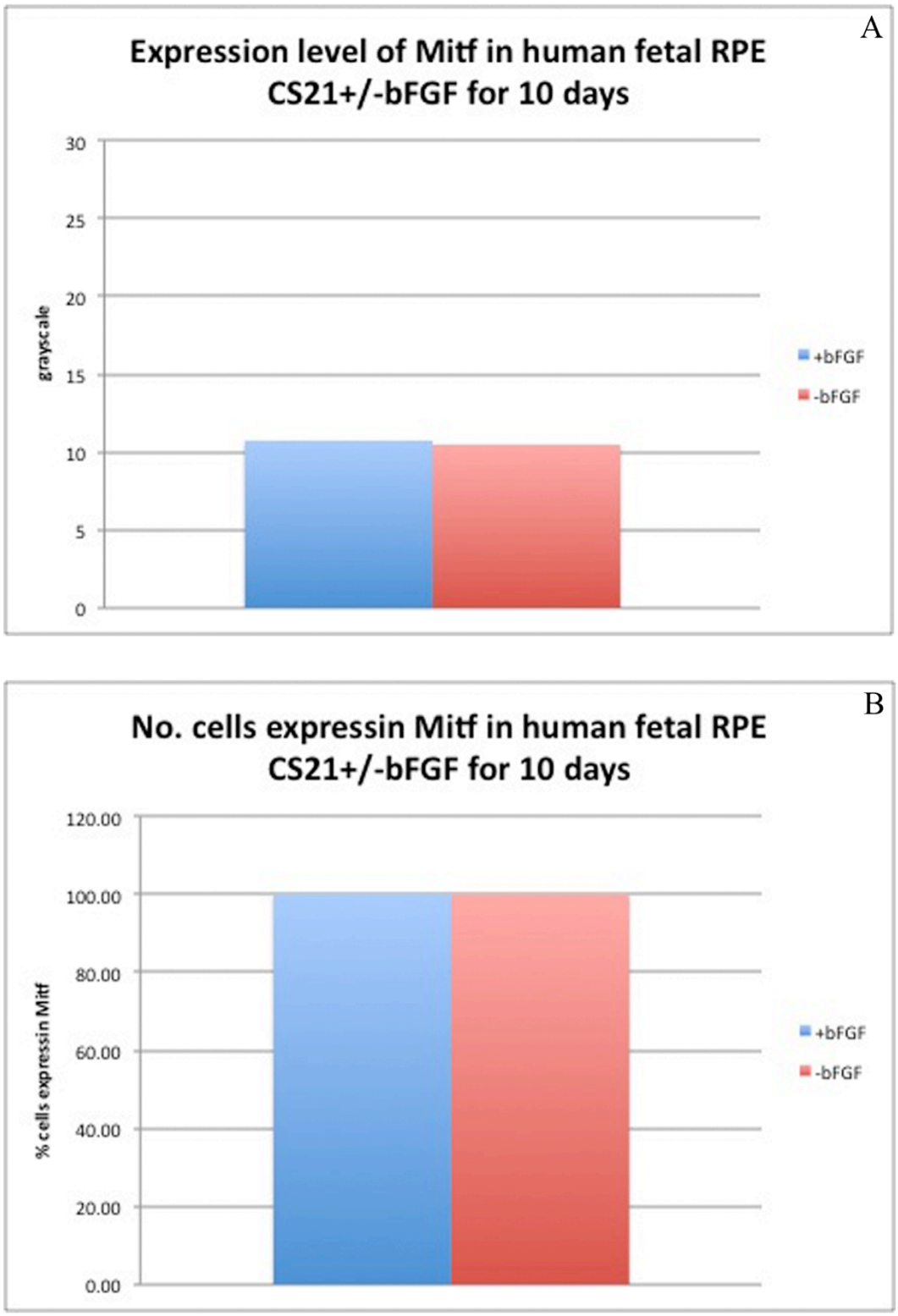


Fig. 5.12
Human fetal RPE CS21 explants +/-bFGF (100ng/ml) for 10 days.
(A) quantification of the average pixel intensity of grayscale images of Mitf expression images for each explant +/-bFGF. These were calculated by thresholding and quantification of pixel intensity of the fluorescent signal using image analysis software. Expression level of Mitf is comparable in either +/- bFGF condition. (B) Number of cells in each explant +/-bFGF expressing Mitf by counting. All cells in either condition were observed to express Mitf. This suggests that no transdifferentiation has taken place.

Given the apparent disorganization of multi-layered cells in the explant treated with bFGF, it was thought that this may indicate that the cells are proliferating in response to the exogenous growth factor. However, immunohistochemical labeling for Ki67, a proliferative cell marker, did not reveal any marked difference between the two culture conditions (Fig. 5.14A, B). Given the lack of tissue required to repeat the experiment, a grading system was used to compare the numbers of Ki67 positive cells given that exact figures may be mis-leading. Both treated and untreated explants were categorized as grade 1, displaying less than 5 cells in an entire section (Fig. 5.15B). Only a few cells in either condition exhibited nuclear labeling for Ki67, with 2 positive cells observed in the untreated explant (Fig. 5.14A yellow arrow), and 1 positive cell in the +bFGF explant (Fig. 5.14B yellow arrow). The average expression level of Ki67 in either condition was observed to be relatively comparable, with only a very slight increase in expression of Ki67 in the explant treated with bFGF, compared with the negative control (n=1) (Fig. 5.15A). Co-localisation of Ki67 with Pmel17 (Fig. 5.14 A, B), as well as the fact that the cells are pigmented (Fig. 5.14 C, D) indicates that these Ki67 positive cells are RPE cells which have retained their characteristic phenotype. The +bFGF treated RPE explant displays weaker pigmentation than the untreated negative control as previously discussed (Fig. 5.14 C, D).

If transdifferentiation towards a neuronal phenotype was to occur in response to exogenous bFGF treatment, the likelihood is that cells would express early neuronal markers such as nestin and glial fibrillary protein (GFAP), which are expressed early in retinal development. No GFAP expression was observed in either bFGF treated or untreated explants (data not shown), however, some nestin expression was present in the bFGF treated RPE explant, but was absent from the RPE in the negative control (Fig. 5.16). Nestin expression was observed in both conditions within the extra-ocular tissue attached to the basal side of the RPE (Fig. 5.16A, B, E, F, G, H), however, nestin expression did not co-localise with the pigmented, Pmel17 positive RPE cells on the surface of the aggregate in untreated cells (Fig. 5.16A, E, G).

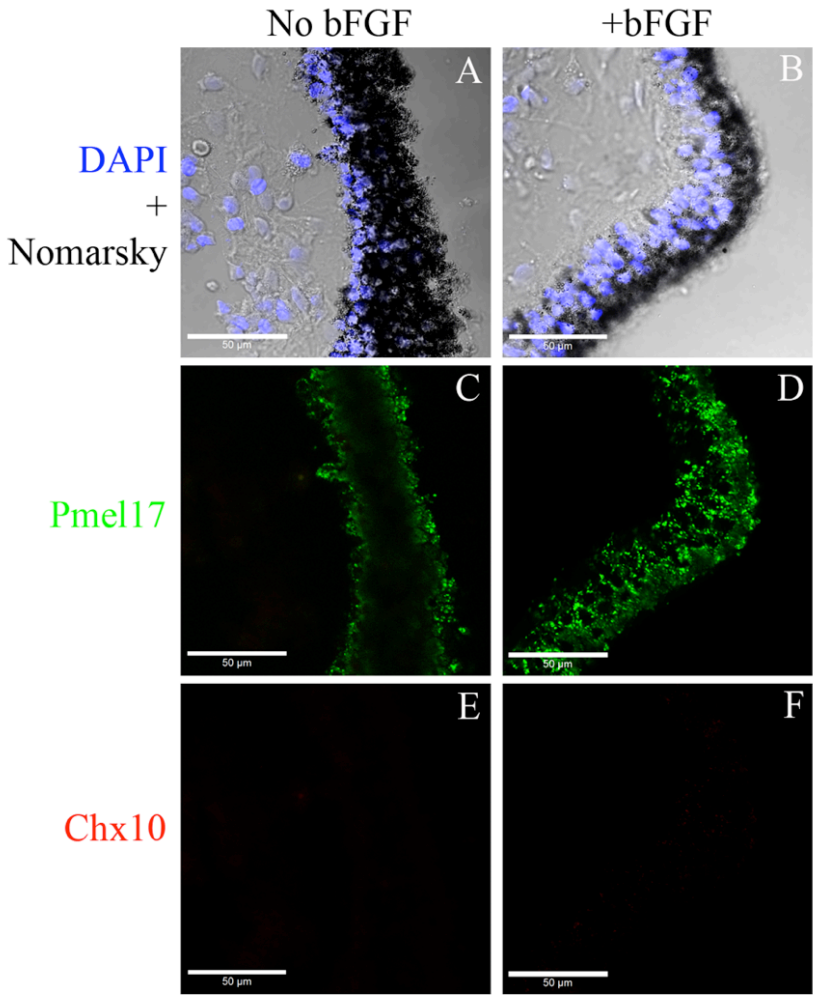


Fig. 5.13
Expression of Pmel17 (green) + Chx10 (red) in human fetal RPE CS21 explants treated +/-bFGF (100ng/ml) for 10 days.
Both bFGF treated, and untreated explants retained their pigmentation (A, B). Untreated RPE retained its characterisitic epithelial morphology (A), whereas bFGF treated aggregates appeared to exhibit multiple layers of cells (DAPI, blue) (B). Both untreated (C) and bFGF treated eplants (D) expressed Pmel17 in a granular fashion, however, Chx10 was not observed in either culture condition (E, F). This suggests a lack of transdifferentiation in response to bFGF. Scale bars: 50uM.

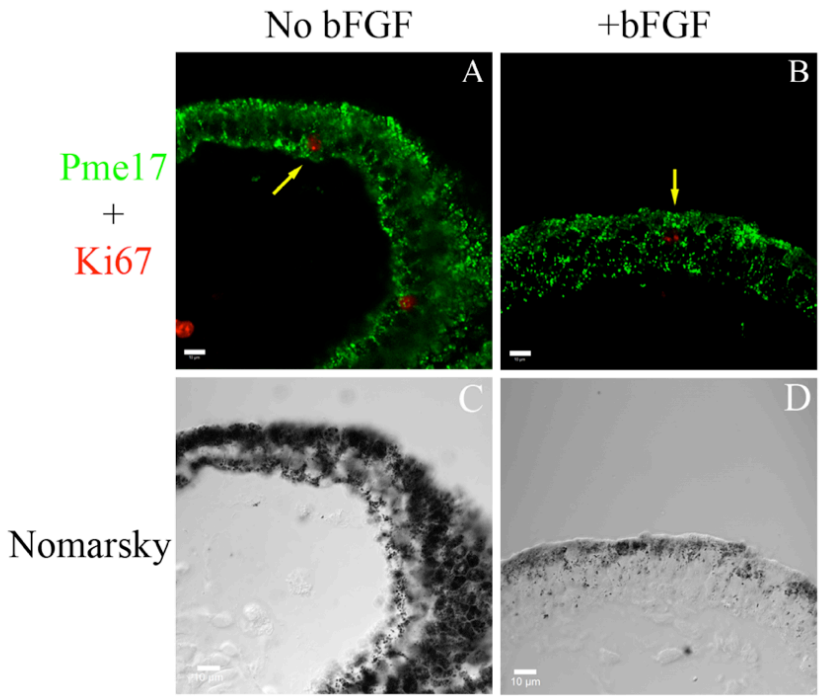
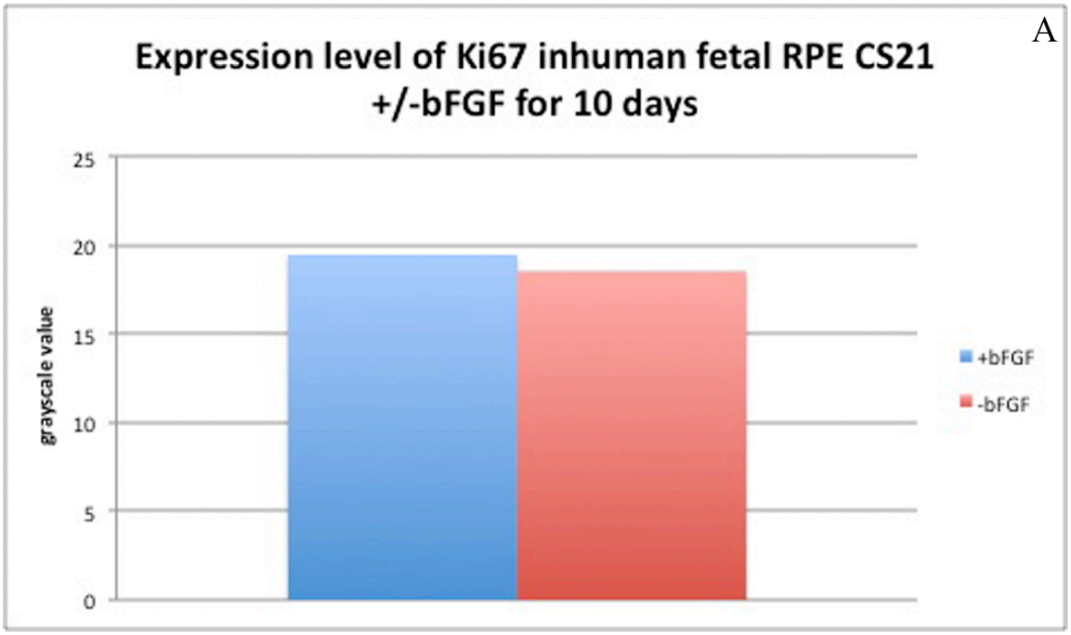


Fig. 5.14
Expression of Pmel17 (green) + Ki67 (red) in human fetal RPE CS21 explants +/- bFGF (100ng/ml) for 10 days.
-bFGF (A, C), +bFGF (B, D). Pmel17 expression was observed in both +/-bFGF culture conditions (A, B), as was pigmentation (C, D). Pigmentation appeared to be reduced in bFGF treated explants (D) in comparison to untreated negative controls (C). Very few cells were proliferative in either condition as demonstrated by the low number of cells expressing Ki67 (red) (A, B yellow arrows). Ki67 positive cells co-localised with Pmel17 which suggests that is is the RPE cells which are dividing and not another, contaminating cell type. Scale bars: 10uM

A



B

Treatment	Ki67 grade
+bFGF	1
-bFGF	1

Grading System			
0	=	0 cells	None
1	=	1-5 cells	Very few
2	=	6-10 cells	Few
3	=	11-20 cells	Moderate
4	=	21-30 cells	Many
5	=	31+ cells	Most

Fig. 5.15
Expression of Ki67 (red) in human fetal RPE CS21 explants +/-bFGF (100ng/ml) after 10 days.
(A) the average pixel value of Ki67 expression in whole images +/-bFGF shows a slight increase in expression in the bFGF treated explant compared with the untreated control (n=1), however, the difference is minimal, and it is likely that the levels are comparable. (B) Scoring of the number of cells expressing Ki67 in each section of each explant in either +bFGF or -bFGF culture conditions. Both culture conditions were given a score of 1, which shows that the numbers of cells expressing Ki67 in either condition are comparable. Very few cells express Ki67 in explanted RPE cultures following 10 days.

In contrast, nestin expression in the explant treated with bFGF did co-localise with RPE marker Pmel17 (Fig. 5.16B, F yellow arrow) as well as pigmentation (Fig. 5.16H, J), in a cell layer that appeared to be thicker and less epithelial in appearance than the negative control. Nestin expression within RPE cells treated with bFGF was not found in all cells of the explant, but was confined to a small region of Pmel17 positive cells (Fig. 5.16B white box). Other regions of the bFGF treated explant were negative for the neuronal marker (Fig. 5.16B), despite some of them appearing much less pigmented and thicker than the nestin positive region (Fig. 5.16D), characteristics usually associated with areas of transdifferentiation. As previously discussed, the bFGF treated explant did appear to have a lower level of pigmentation (Fig. 5.16B) than the negative control (Fig. 5.16A). Nestin positive cells displayed labeling for the protein with a fibrillar pattern within their cytoplasm, which is consistent with the pattern of intermediate filaments in neuronal cells. It is also clear that the levels of pigmentation are heterogenous within both treated (Fig. 5.16D), and untreated explants (Fig. 5.16C), however, it appears as though the contrast in differing pigmentation levels is greater in bFGF treated cells. Image analysis of nestin expression in *regions of* interest in the low magnification images shown in Fig. 5.16A and B, show that there is a higher expression of nestin in bFGF treated explants than untreated explants, however this difference is not statistically significant (n=4, RANOVA) (Fig. 5.17A). Despite this, the low resolution of low magnification images, in addition to the fact that the regions of interest do not account for the largely regionalized positive expression of nestin. Therefore, a similar quantification analysis of the high magnification image of nestin expression in RPE cells (Fig. 5.16G, H) was undertaken to highlight the contrast in expression between bFGF treated, and the untreated (nestin negative) control. This clearly shows the large difference in the expression level of nestin within Pmel17 positive RPE cells (Fig. 5.17B) of bFGF treated explants in comparison to the negative control. A scatterplot of the regional expression of nestin, when compared with the corresponding level of Pmel17 expression in the same region, demonstrates that there is little relationship between the level of Pmel17 expression and nestin expression (Fig. 5.18A).

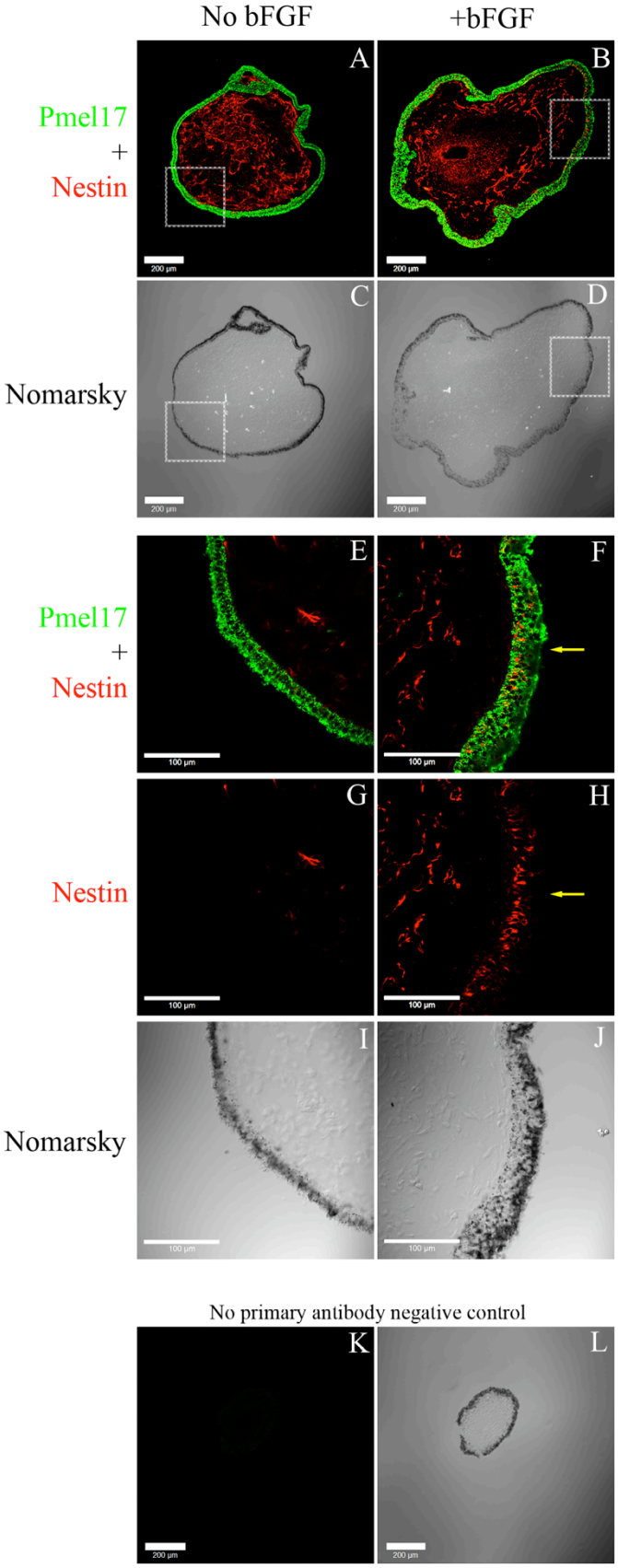


Fig. 5.16
Expression of Pmel17 (green) + Nestin (red) in human fetal RPE explants CS21 treated with bFGF (100ng/ml) for 10 days.
-bFGF (A, C, E, G, I), +bFGF (B, D, F, H, J). Expresison of Pmel17 was present in all cells of both bFGF treated and untreated explants (A, B, E, F). Nestin expression was observed in both culture conditions, however, this was largely confined to he extraocular tissue attached to the basal surface of the explanted RPE (A, B, E, F, G, H). The bFGF treated aggregate expressed nestin in one region of Pmel17 positive, explanted RPE (B white box, high mag. in F, H yellow arrows) whereas it was absent from the untreated control (A white box, high mag. in E, G). The corresponding level of pigmentation in this region of nestin expression was equivalent to that of the negative control (C, D white boxes, high mag. in I, J), however, bFGF treated RPE appeared to be a number of cells thicker, and more disoranised (J) than the single layer of pigmented RPE in the untreated explant (I). Despite this, the level of pigmentation varied in different regions throughout the explants, in both culture conditions (C, D). (K, L) no primary antibody controls negative for secondary labeling. Fluoresence digitally enhanced. Scale bars: (A-D, K, L) 200uM, (E-J) 100uM.

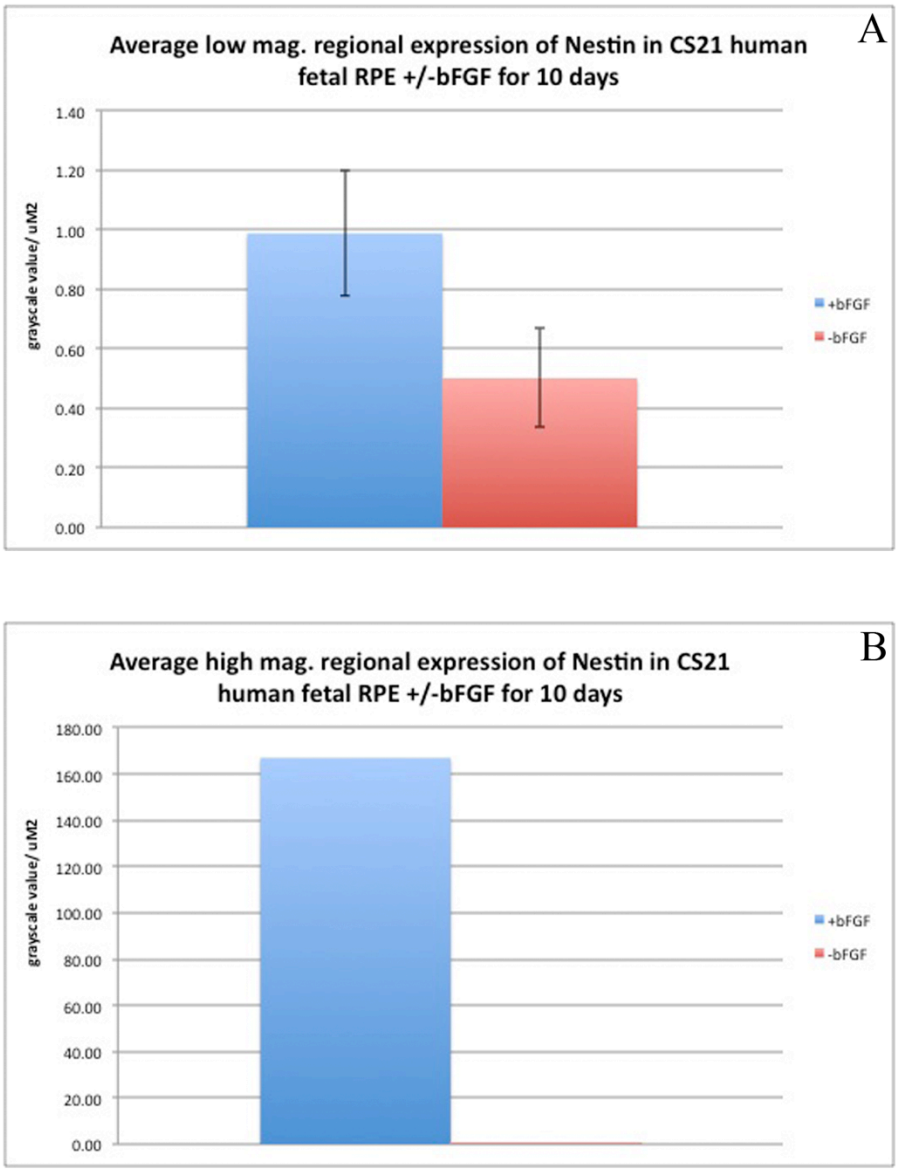
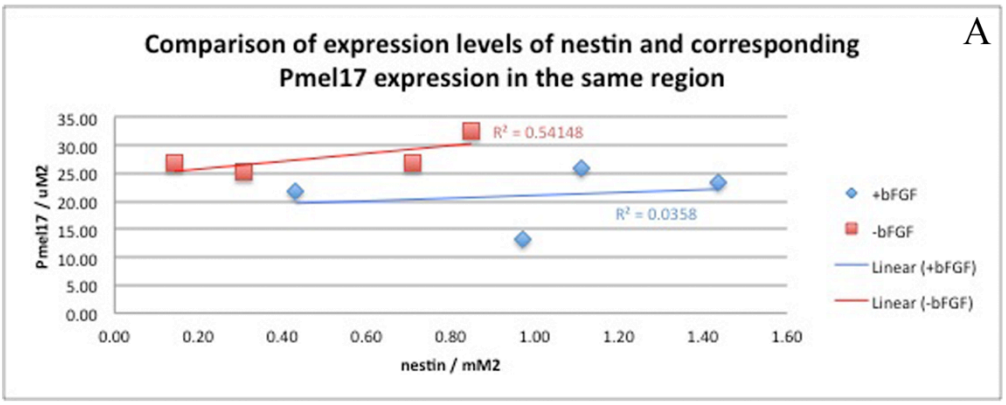


Fig. 5.17
Expression levels of nestin in human fetal RPE CS21 explants +/-bFGF after 10 days.
(A) average pixel intensity of grayscale images displaying nestin expression in 4 regions of explanted RPE +/-bFGF from a low magnification image (RPE derived from same embryo, n=1). Nestin expression is significantly higher in the RPE of the bFGF treated explant than the untreated, negative control. (B) average pixel intensity from high magnification images of the same explants +/-bFGF highlighting the more easily observable difference in nestin expression at higher resolution (n=1, where n is the number of meaasurements for each condition).

This appears to be the case for both bFGF treated and untreated explants given that the trendlines for both conditions appear relatively horizontal, with perhaps a slight positive incline, however, given the nature of the small increments between the different levels of nestin expression, this is not likely to be significant. The R^2 values for the trendlines of both culture conditions were relatively low, with $R^2 = 0.54$ (n=4) for –bFGF, and $R^2 = 0.04$ (n=4, where n = No. of regions of interest) for +bFGF treated explants (Fig. 5.18A). Similarly, both trendlines for a scatterplot comparing nestin expression with the corresponding level of pigmentation were similarly horizontal, with the trendline for +bFGF explants appearing the have a slight negative correlation, implying higher expression of nestin in more pigmented regions, whereas –bFGF explants exhibited a slight positive incline, implying higher nestin expression in lighter regions (Fig. 5.18B). However, the R^2 value for the +bFGF treated explant was low at $R^2 = 0.2986$ (n=4, n = No. of regions of interest), therefore suggesting a very weak correlation. The R^2 value for –bFGF cultured explants implied a strong, slightly positive correlation, with a value of $R^2 = 0.93825$ (n=4, where n = no. regions of interest). This positive correlation would suggest a rapid change in pigmentation with very little change in nestin expression. It is therefore possible that this is an artifact of the low sampling number.

Given the fact that RPE have been reported to transdifferentiate towards a lens phenotype in response to bFGF, in addition to producing new neurons, it was necessary to investigate the expression of the major structural protein of the lens, αA-crystallin. Both explants were observed to express αA-crystallin, independently of the treatment with bFGF (Fig. 5.19A, B; E, F yellow arrows). The positively labeled cells displayed a fibrillar pattern of expression throughout the cytoplasm of pigmented RPE cells which were also positive for Pmel17 expression (Fig. 5.19E-H yellow arrows). Cells positively labeled for αA-crystallin were scattered throughout the RPE cell layer rather than being confined to a particular region (Fig. 5.19A, B). Quantification of the relative expression levels of αA-crystallin in either culture condition suggested that αA-crystallin expression was lower in the explant treated with bFGF (n=4, where n is the no. of regions of interest) when compared to the untreated negative control explant (Fig. 5.20).



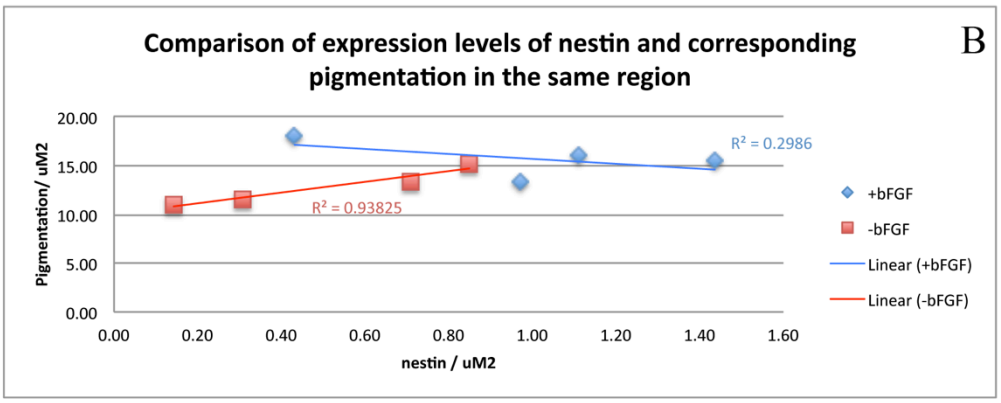


Fig. 5.18
Comparison of the level of nestin expression with corresponding Pmel17 expression (A), and levels of pigmentation (B), in 4 regions of interest of human fetal RPE CS21 +/-bFGF for 10 days. Several regions of interest were selected in multi-channel fluorescence/brightfield images. It was therefore possible to measure the average pixel intensity of fluorescence/pigmentation in each region, for the same corresponding regions of each channel, from a single image. By using this system it was possible to analyse the potential relationship between localised expression patterns of different markers/pigmentation. (A) Weak if any correlation exists between the expression of Pmel17 and nestin, regardless of treatment with bFGF or not. (B) Simiarly, little correlation between nestin expression and the level of pigmentation is apparent for the explant rteated with bFGF. A strong positive correlation exists for theuntreated explant, however, ths may be a result of the low sampling number. Grayscale: Black = 0, White = 255.

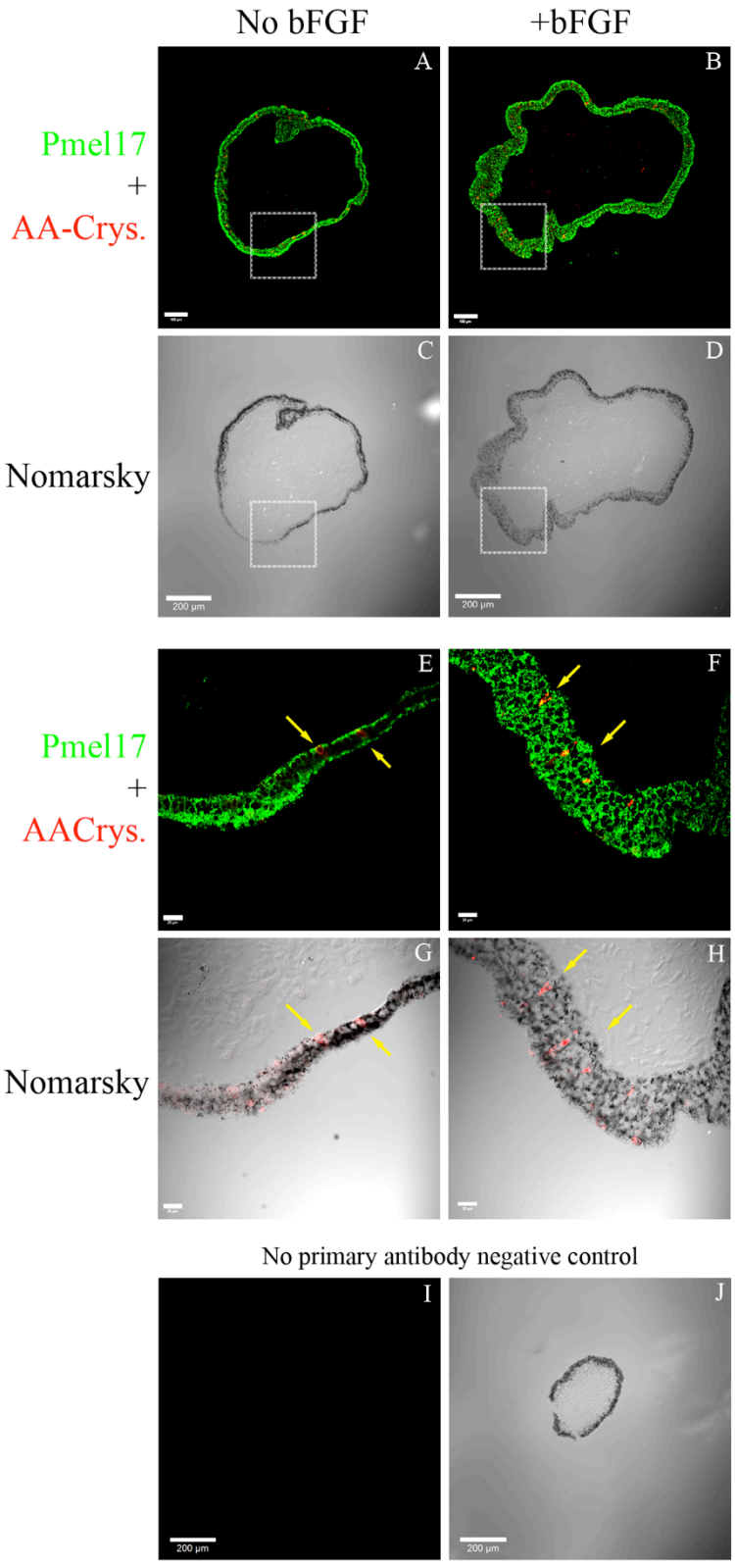


Fig. 5.19
The expression of Pmel17 + alpha-A-Crystallin in human fetal RPE CS21 treated with bFGF (100ng/ml) for 10 days. Pmel17 (green) + alpha-A-crystallin -bFGF (A), +bFGF (B). alpha-A-Crystallin found throughout explants treated both with and without bFGF. (E-H) high magnification images of white boxes in A, B respectively. alpha-A-Crystallin found in the cytoplasm of explanted Pmel17 positive, pigmented RPE cells (E-H, yellow arrows). Explant treated with bFGF appears to have thicker, mulit-layered regions (B, D, F, H) than untreated explant (A, C, E, G). Explant in both conditions displayed variable pigmentation throughout (C, D). No signal in nom primary antibody negative control (I, J). Fluorescence digitally enhanced. Scale bars: 20uM (A, B, E-J), 200uM (C, D).

However, the relative difference between the levels of expression of αA-crystallin in either condition was very small and was not statistically significant (n=4, RANOVA). The level of expression of αA-crystallin did not strongly correlate with the expression of RPE marker Pmel17, despite the fact that an almost identical positive gradient was observed for both culture conditions (Fig. 5.21A). The R^2 values for both +bFGF and –bFGF treated explants were low at $R^2 = 0.33439$ and $R^2 = 0.16918$ respectively (n=4, where n is the no. regions of interest for each culture condition), which suggests that any observed correlation is very weak. Similarly, little correlation was observed between αA-crystallin expression and the level of pigmentation, with similarly, horizontal trendlines, in addition to low R^2 values of $R^2 = 0.38618$ and $R^2 = 0.07942$ for –bFGF and +bFGF culture conditions respectively (Fig. 5.21B).

Several of the human fetal RPE sections appeared to be thicker when treated with bFGF when compared with their untreated control, despite retaining their characteristic pigmentation (Figs 5.11, 19). Additionally, the explant treated with bFGF often appeared to be multi-layered and less epithelial in appearance than untreated controls. Given the fact that a loss of epithelial phenotype and thickening of the RPE are characteristics of transdifferentiating RPE monolayers, it was necessary to quantify the average thicknesses of each section in order to ascertain whether or not bFGF was responsible for thickening of the RPE. The average width of RPE the RPE explant treated with bFGF was observed to be thicker than that of the untreated negative control (Fig. 5.22), and this difference was observed to be statistically significant ($p<0.05$, n=12, where n is the number of thickness measurements, RANOVA).

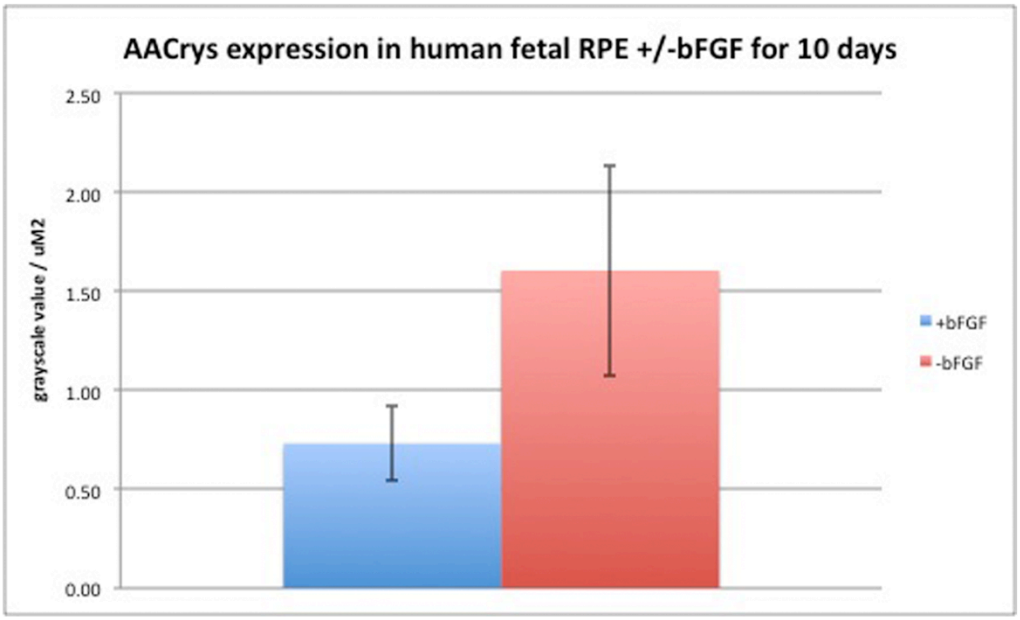
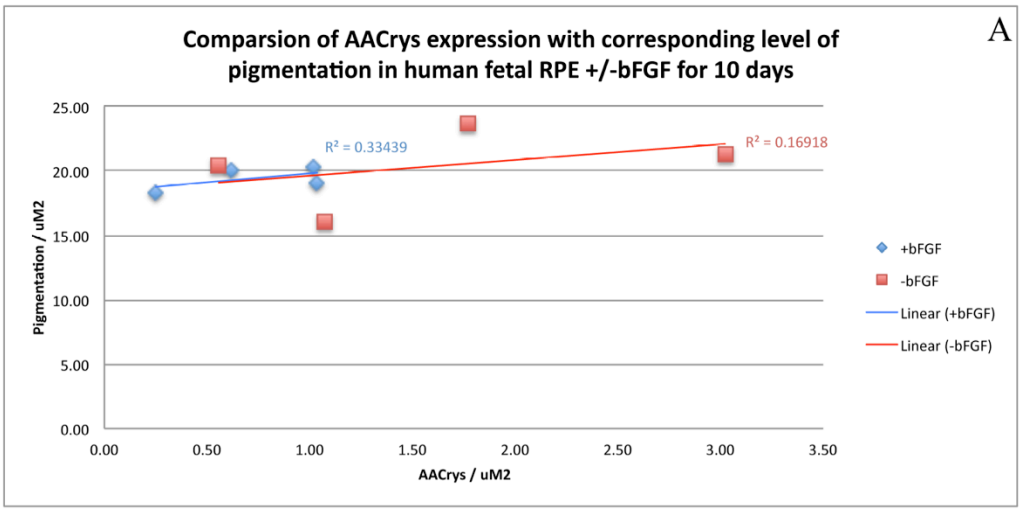


Fig. 5.20
Quantified expression of alpha-A-Crystallin in human fetal RPE CS21 +/-bFGF (100ng/ml) for after 10 days in culture.
The average pixel intensity of fluorescence was calculated from 4 regions of interest in thresholded, grayscale images using image analysis software. Thresholding was employed to only measure positive fluorescence pixels and not negative background pixels. alpha-A-Crystallin is significantly higher in untreated explants than in bFGF treated explants (n = 4, where n is the number of regions of interest). The reason for this is unclear, however, it may indicate that the untreated RPE exists in a more stessed state than that treated with growth factor bFGF.



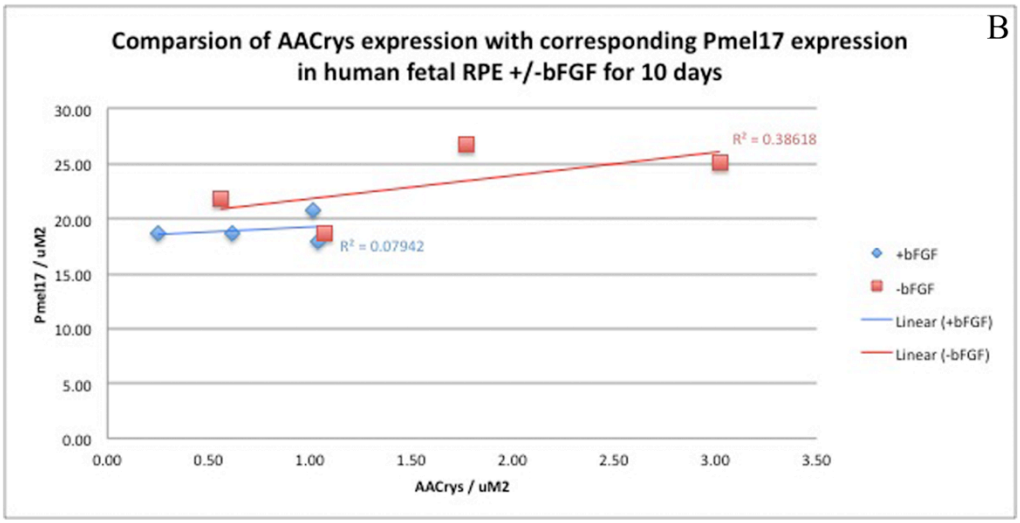


Fig. 5.21
Comparison of the expression of alpha-A-Crystallin with the corresponding level of pigmentation (A), and the level of expression of Pmel17 (B) in regions of interest (n = 4) of human RPE CS21 explants +/bFGF after 10 days.
The average pixel intensity was measured in thresholded grayscale images using image analysis software. In order the analyse potential correlation in the localised expression of fluorescence/pigmentation, 4 regions of interest were selected for each multi-channel image. The quantification of the average pixel intensity in each region, in each single-channel of fluorescence/pigmentation means that the same region is measured for each corresponding channel. (A) A slight positive incline of the trendline is observed for a plot of the comparison of alpha-A-crystallin with the level of pigmentation in both culture conditions, suggesting that alpha-A-crystallin expression decreases with pigmentation. However, very low R2 values suggest that no strong correlation exists. (B) Similarly, low R2 values exist for both +/-bFGF explants when comparing alpha-A-Crystallin and Pmel17 expression, which suggests little, if any correlation between the expression levels.

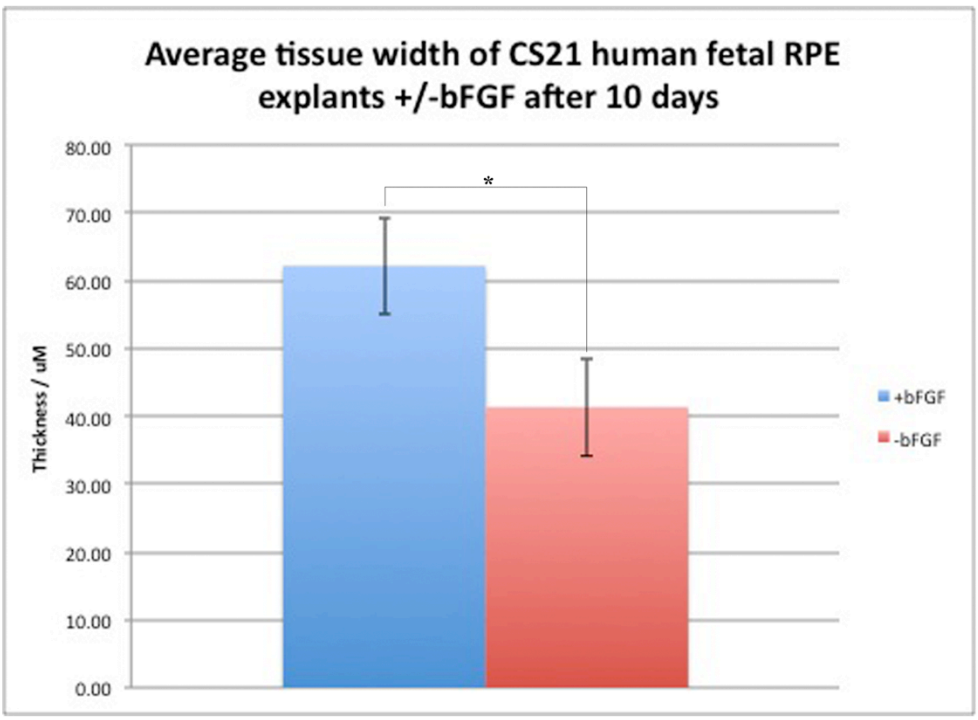


Fig. 5.22
The average thickeness of sections of human fetal RPE CS21 +/-bFGF (100ng/ml) after 10 days.
The width of the RPE monolayer of each low magnification image (encompassing the entire section of each explant) was measured at 4 different points around the circumference of the sections. These measurements included the both the thickest, and thinnest parts of the explant. An anverage of these measurements was calculated for each image to give an average width of the tissue for that image. The averages for all sections were subsequently averaged to give a final value. bFGF treated explants are significantly thicker than untreated explants ($p<0.05$, $n = 16$, where n is the number of thickness measurements, RANOVA). This is a feature normally associated with the induction of transdifferentiation. Error bars: Standard Error.

5.2.4 Discussion:

The induction of transdifferentiation in response to bFGF has been shown to be dependent on FGF/FGFR/Mek/Erk –mediated up-regulation of Pax6 (Spence et al., 2007b). It has been suggested that this up-regulation of Pax6 in response to bFGF is as a result of a down-regulation of RPE transcription factor, Mitf (Nguyen and Arnheiter, 2000). Both Pax6 and Mitf have been shown to have the ability to regulate one another's expression levels in a reciprocal manner (Fuhrmann et al., 2000b, Iwakiri et al., 2005, Mochii et al., 1998a), and it has been suggested that a loss in Mitf expression results from a MEK-1 dependent pathway in a similar manner to that of Pax6 (Spence et al., 2007b). Certainly, a loss in Mitf expression alone is not sufficient for the induction of transdifferentiation, as it was reported that removal of the retina in a chick embryo is sufficient for spontaneous down-regulation in Mitf expression (Spence et al., 2007b). However, it remains to be seen whether or not this would also be the case in chick RPE explants (Liu et al., 2009b). Mitf expression is also routinely down-regulated following dissociation of RPE cells which induces de-differentiation and proliferation of the cells (Liu et al.). This phenomenon has been associated with proliferative vitreoretinopathy (PVR), the scarring which can occur as a result of damage to the RPE via a process known as epithelial-mesenchymal-transition (E-M-T).

It is reported that a bFGF-dependent increase in Pax6 expression can itself down-regulate the expression of Mitf (Spence et al., 2007b). It is possible that this occurs through direct interaction of Pax6 with Mitf transcription machinery, and/or as one component of a more complex network of transcriptional changes responsible for initiation of transdifferentiation of the RPE. As a result, Pax6 is often referred to as the master transcriptional regulator of transdifferentiation given its central role in both inducing the phenomenon, as well as maintaining the on-going development of the resulting neural retina, through its function as the 'master eye gene' (Azuma et al., 2005a, Ziman et al., 2001, Chow et al., 1999, Philips et al., 2005, Weasner et al., 2009, Zuber et al., 2003).

The absence of Pax6 in human fetal explants cultured both with and without bFGF, in conjunction with retention of Mitf expression, would therefore suggest that RPE explants did not undergo transdifferentiation towards a neuronal phenotype in response to bFGF. This is because Pax6 appears to be crucial for both the induction and maintenance of transdifferentiation, as well as early retinal development (Spence et al., 2007b, Nishina et al., 1999, Sakami et al., 2008). It is unclear at this stage whether or not the absence of Pax6 from explants results from a down-regulation of the transcription factor in RPE cells prior to dissection at this particular developmental stage (CS21, 8 weeks), as is reported to be the case in other species (Spence et al., 2007b), or whether the culture system itself was responsible for a down-regulation of the protein. What is clear is that regular dosage with a high concentration of bFGF (100ng/ml) was not able to either induce, or maintain Pax6 expression in CS21 RPE cells in this culture system. It has been reported that the presence of Pax6 expression in RPE cells is necessary for bFGF-mediated transdifferentiation to occur in amphibians (Kuriyama et al., 2009a), and a loss in expression of the transcription factor coincides with the loss in capacity for transdifferentiation in response to bFGF in the embryonic chick (Spence et al., 2007b). This suggests that the maintenance of Pax6 expression is important for bFGF to initiate transdifferentiation. In keeping with this, over-expression of Pax6 in the RPE of chick embryos can itself trigger transdifferentiation independently of exogenous addition of bFGF (Azuma et al., 2005a). Interestingly, this process is reported to induce transdifferentiation at much later stages of development than bFGF has the capacity to, albeit with a progressively reduced efficiency. The efficiency of transdifferentiation in response to ectopic Pax6 is reported to decrease with increasing developmental stage of the embryo (Azuma et al., 2005a). It remains unclear whether or not RPE cells would react in a similar manner *in vitro,* and therefore, it is currently unclear whether Pax6 over-expression directly induces transdifferentiation, or merely sensitizes the RPE to an environmental cue which is already present/is induced through interaction with the Pax6 expressing RPE cell. If the former is true, this would suggest that the restriction in the

capacity for transdifferentiation in response to bFGF at later developmental stages occurs upstream of Pax6.

Chx10 is also known to have an antagonistic relationship with RPE-related transcription factor Mitf, where an increase in expression levels of either protein can negatively affect the expression of the other (Fuhrmann et al., 2000b, Horsford et al., 2005, Rowan et al., 2004, Bharti et al., 2008). An increase in Chx10 expression in the developing retina is associated with a loss of pigment and the development of a multi-layered presumptive neuroretinal morphology (Muller et al., 2007, Fuhrmann et al., 2000b, Fujimura et al., 2009, Spence et al., 2004), in addition to the expression of other retinal progenitor markers. It is possible that the observed down-regulation of Mitf, via an up-regulation of Pax6 in transdifferentiation, could occur through an up-regulation of Chx10. Chx10 would then subsequently inhibit Mitf expression and contribute to the induction of a neuroepithlial phenotype. It may be that Pax6 is able to interact with the transcription machinery of Mitf both directly (Baumer et al., 2003), and indirectly via up-regulation in Chx10 (Fuhrmann et al., 2000b, Bernier et al., 2001). The absence of Chx10 expression in explants treated with or without bFGF would suggest that no RPE to neuroretinal transdifferentiation has taken place, given that the protein appears to be crucial for a suppression of the RPE phenotype, and the progression of early retinal development.

Consistent with this idea, the expression level of Mitf appears to be relatively consistent across all cells in both untreated, and bFGF treated human fetal RPE explants, which would again suggest that the RPE phenotype has been maintained regardless of the non-adherent culture system, or treatment with bFGF. One would expect at least a decrease in the level of Mitf expression in response to bFGF if transdifferentiation was being initiated. However, the low sampling number for Mitf expression means that a despite an apparently consistent level of Mitf expression across explants in both culture conditions, it is not possible to calculate the error in these values obtained via image analysis. Until the experiment can be repeated with more biological repeats, this result will remain somewhat unreliable.

In addition to the retention of a key RPE transcription factor Mitf, a protein downstream of Mitf, Pmel17, which is important in pigmentation of RPE cells (Baxter and Pavan, 2003), is retained in both bFGF treated, and untreated explants. Unsurprisingly given the expression of Pmel17, the characteristic pigmentation of RPE cells was also present in both culture conditions. This again supports the idea that the RPE phenotype has been maintained and that no transdifferentiation has taken place. As one might therefore expect, quantification of the expression of Pme17, as well as the level of pigmentation, in low magnification images of whole sections of each explant, revealed no significant difference in either marker between bFGF treated, and untreated culture conditions. However, high magnification images of the same sections revealed no significant difference in Pmel17 expression between +/-bFGF treated explants, but there was a statistically significant decrease in pigmentation in the bFGF treated explant compared with the untreated negative control. It is unclear why there is a discrepancy between the low magnification, overall average results, and the higher magnification specific region results. It is possible that the higher magnification images are of a better resolution to accurately ascertain subtle changes in pigmentation in the explanted RPE cells, and therefore highlight a significant change in response to bFGF. However, the difference could be an artifact of the fact that higher magnification images happen to be biased towards less pigmented regions of the explants, given the observed variability of pigmentation throughout each section, compared with the lower magnification images which measure an average value for each whole section. The reason for this difference is unclear and will require further biological repeats of the experiment (should the human fetal RPE tissue become available) in order to confirm any difference in pigmentation between culture conditions. Given the involvement of Pmel17 in the pigmentation of melanocytes, one would expect Pmel17 expression to correlate with the level of pigmentation in the RPE cells, however, this was found not to be the case when a number of different regions of each explant were analysed for the level of Pmel17 expression, and corresponding pigmentation. It is unclear why this would be given the biological relationship reported between the two variables, however, it may be that different stages of

melanogenesis produce differences in the observable levels of pigmentation, and therefore, no obvious correlation between Pmel17 and the level of pigmentation is easily identified. Other investigators have also reported a lack of correlation between Pmel17 and pigmentation, which suggests that this result is consistent with those in other experiments (personal communication). There appeared to be a certain amount of clustering of datapoints for treated and untreated explants, and therefore, it was necessary to analyse the average regional levels of expression, of each marker, in each culture condition, to see if these averages were consistent with whole explant averaging. Histograms of the regional averages confirmed the fact that no significant differences in Pmel17 expression or level of pigmentation were apparent between bFGF treated, and untreated explants from the low magnification images, which was consistent with whole section averaging discussed earlier. The large standard error bars for these graphs serve to highlight the variability the expression of Pmel17 and pigmentation in different areas of each explant. If the de-pigmentation in response to bFGF is real, then considering the apparently consistent expression of Mitf between bFGF treated and untreated explants, this could suggest the involvement of a Mitf independent pathway able to relay the bFGF signal, and subsequently reduces the level of pigmentation. This pathway would most likely involve other Mitf effectors, downstream of the transcription factor, which are also associated with producing the pigmented RPE phenotype.

Interestingly, the bFGF treated explant appeared to be thicker than the untreated negative control, in addition to having an apparently more disorganized, less epithelial, multilayered appearance in many places. Measurement of the thickness of the pigmented layer in several sections of each explant revealed that the bFGF treated explant was indeed thicker than its untreated control, and that this difference was statistically significant. Thickening of the RPE monolayer is a classical characteristic of transdifferentiation towards a neuroepithelial phenotype (Pittack et al., 1997, Pittack et al., 1991, Park and Hollenberg, 1989, Park and Hollenberg, 1991), however, this is usually associated with proliferation (Stroeva and Mitashov, 1983) and de-pigmentation of cells as they expand and re-differentiate as neuronal cells. Investigation into the level of proliferation of cells in each explant revealed

that only very few cells were observed to be undergoing proliferation in either culture condition. One would expect the majority of cells to be proliferative had transdifferentiation taken place, resulting in the formation of new retinal progenitors. It is possible that cells had undergone proliferation earlier in the culture period in response to bFGF, resulting in the formation of these multilayered structures, and subsequently become quiescent after the full 10 day culture period. It would not be possible to confirm this hypothesis without further repetition of the experiment, requiring more rare tissue. It was thought that the morphogenic properties of bFGF could been responsible for the multilayered structures observed, as a result of proliferation of RPE cells, however, a lack of significant proliferation would suggest that this is not the case.

However, it may be that if more biological repeats were available, bFGF treated explants would not be observed to be thicker than untreated explants. If +bFGF treated explants were still found to be thicker, it would be necessary to either pulse cells with BrdU and see whether or not this was incorporated into cells of the explant after 10 days, or alternatively, sample the cultured RPE explants at a number of stages earlier than 10 days. The lack of proliferation, coupled with the expression of RPE markers and pigmentation, would most likely suggest that these apparently multilayered, thicker regions are a result of tangential sectioning for the +bFGF explant, in comparison to a more cross-sectional section in the control.

In order to investigate the capacity for RPE explants to express neuronal markers in response to bFGF, as would be the case in transdifferentiating RPE, both nestin and GFAP expression were investigated. GFAP was absent from both bFGF treated and untreated RPE explants, which is not unexpected given the clear retention in the characteristic RPE phenotype of the explants. However, neuronal progenitor marker nestin was expressed in the RPE explant treated with bFGF only. This expression was co-localised with intense pigmentation and Pmel17 expression, showing that the RPE cell component of the explant is responsible for this signal, in comparison to the general signal detected in the Pmel17/pigmentation negative, extra-ocular tissue on the basal side of both bFGF treated and

untreated explants. Despite this nestin expression, no other obvious characteristics of transdifferentiation were observed. Interestingly, the nestin expression apparently induced by bFGF treatment was largely confined to one region of the explant, with no positive cells present in other regions. This is reflected in the quantification of expression from a low magnification image of a whole section, where an average of 4 different regions of interest shows a small, increased level of nestin expression than the background for untreated, negative control explants. This difference was not observed to be statistically significant, probably as a result of the negative regions lowering the overall average value for the calculation over a number of regions of interest. The difference is more distinct when the level of expression of a high magnification image of the nestin positive region in the bFGF treated explant, is compared to the negative untreated control. However, lack of biological repeats means that no standard error or statistics can be calculated for the significance of this difference. In order to prove the significance of this difference in expression, once again more repetitions of the experiment would be required but limitations in the supply of this valuable tissue means that this would be very difficult. Nevertheless, immunohistochemical analysis does strongly suggest that nestin expression is dependent upon exogenous bFGF treatment, which suggests that RPE cells at this stage of development can respond to bFGF treatment to a degree, even if full transdifferentiation towards a neural retinal neuroepithelium is not observed. This is important given that it has been suggested that a loss in the expression of the bFGF receptor, FGFR-1, and therefore a loss in the transduction of the bFGF signal, is responsible for a loss in the capacity of RPE cells to undergo transdifferentiation in response to bFGF (Spence et al., 2004). It is possible that bFGF is able to activate a portion of the signaling cascade responsible for neuronal specification in RPE cells, and therefore initiate an early marker of neuronal development in nestin, but other signaling components required for full transdifferentiation are still inhibited. If this is the case, the identity of these inhibitory mechanisms would likely be vital to unlocking the potential for transdifferentiation of RPE cells.

Interestingly, the fact that only a particular region of the RPE was observed to express nestin in response to bFGF may suggest that the RPE sheet is not homogenous in its response to growth factor treatment, but some areas may be more sensitive to the effects of bFGF than others, possibly as a result of different regions of RPE maturing at different rates. If this is true then this would also be a key factor in understanding the mechanism of control for transdifferentiation.

The region of the bFGF treated explant strongly expressing nestin did not appear to correlate with the most lightly pigmented area of the explant as one might expect of cells losing an RPE phenotype, and re-specifying towards a more neuronal phenotype. This qualitative analysis was backed by a quantitative comparison of nestin expression, with both Pmel17 expression, and levels of pigmentation in different regions, which showed no strong correlation between nestin expression and Pmel17/pigmentation. The only positive correlation observed in these plots was an apparent relationship between lighter regions of the untreated explant and a slightly higher level of nestin expression. However, given the fact that the untreated explant was completely negative for a positive nestin signal, this result is a false positive resulting from the background signal, the apparent correlation possibly a result of fractionally higher background intensity being detected in more lightly pigmented regions.

It is important to note that nestin expression may indeed be present in some RPE cells *in vivo,* and that the observed nestin expression in the bFGF treated explant in this experiment could be an artifact of this which just happens to be absent from the untreated explant. This seems unlikely given robust expression in the bFGF treated explant but it is not possible to rule out whether or not this is the case without further repetition of the experiment, once again requiring more tissue, or indeed investigating the expression of nestin in RPE cells during embryonic development. To our knowledge, no nestin expression has been reported in native, healthy RPE cells at any developmental stage.

The fact that human RPE cells expressed αA-crystallin in the RPE cells, regardless of treatment with bFGF, was surprising as, to our knowledge, this marker has not previously been reported to be expressed outside the lens within the eye. Its presence in RPE cells could

possibly indicate that under these standard culture conditions, these RPE cells are undergoing transdifferentiating towards a lens phenotype. This is possible given that some species are able to regenerate their lens via transdifferentiation of RPE cells in response to various growth factors (Hyuga et al., 1993, Kodama and Eguchi, 1995, Eguchi, 1988, Eguchi, 1986). However, it is also known that members of the crystallin family are able to act as chaperone proteins in addition to their primary functions (Cherian-Shaw et al., 1999, Das et al., 1999, Kundu et al., 2007, Sun et al., 1997, van Boekel et al., 1999, van den et al., 1996). This is often in response to stress where they act as heat shock proteins. Indeed αB-crystallin has been reported to be expressed throughout the retina as well as in the lens itself. This could possibly support the fact that although not statistically significant, a difference in the expression level could be seen between bFGF treated, and untreated control, with a higher expression observed in the untreated explant. This suggest that bFGF is able to suppress the expression of αA-crystallin, possibly as a result of trophic support, and therefore a reduction in the level of stress being experienced by the cells.

The fact that no lentoid structure is visible, and that αA-crystallin expression is less in the bFGF treated explant might suggest that an action as a chaperone is the reason for the presence of crystallin positive cells. It is unlikely therefore that the presence of αA-crystallin in explants is as a result of transdifferentiating RPE cells. This therefore begs the question as to what is responsible for the induction of expression of this protein. It could be possible that the growth medium, itself optimised for growth of stem cells rather than RPE cells, or the non-adherent culture, are responsible for induction of αA-crystallin expression, as a result of the cells experiencing stress in a non-optimal environment. It would be necessary to repeat the experiment in a variety of different culture media in order to confirm this hypothesis. Little correlation between the level of expression of αA-crystallin and the level of Pmel17 expression, as well as the level of pigmentation, suggests that αA-crystallin is not linked to these RPE markers.

If no RPE to neuronal transdifferentiation was observed in human fetal RPE cells in response to bFGF, the question is why? It is possible that human RPE cells are not able to

undergo the same transdifferentiation phenomenon, which has been observed in other species like the embryonic chicken and the rat. However, given the fact that this process appears to be well conserved across a number of different species, this seems unlikely. This is especially unlikely given the capacity for transdifferentiation appears to be a feature of the normal development of the multi-potent cells of the optic cup in these species. These models of bFGF-induced transdifferentiation are reported to lose the capacity at comparable developmental stages, for example embryonic day 5 (E5) in the chicken (Sakami et al., 2008, Park and Hollenberg, 1991, Park and Hollenberg, 1989, Park and Hollenberg, 1993, Pittack et al., 1997, Pittack et al., 1991, Reh et al., 1991), and E15 in the rat (Zhao et al., 1995), which correspond to approximately CS16 (5-6 weeks) in humans. It may be that CS21 human fetal RPE tissue, which is approximately 7 weeks old [which equates to 7.75 days and 16.5 days in chicken and rat respectively (Butler, 1987)] has already lost the capacity to transdifferentiate by this stage. However, results from this investigation (see chapter 3) have demonstrated that transdifferentiation can occur until at least HH27, which still corresponds to an earlier stage of human development (approximately CS18), but is earlier in development than CS21 RPE used here. This begs the question of what is the limiting factor for transdifferentiation in response to bFGF?

It could be possible that the dose of bFGF administered may not have been sufficient to induce human RPE transdifferentiation. However, this seems unlikely given that several studies have reported the onset of the phenomenon following treatment with varying concentrations of bFGF; from 10ng/ml in increments to 100ng/ml. Therefore, the dose used in this investigation seems appropriate given that it is comparatively high compared with other doses reported to have positive effects. It is also worth noting that once the threshold dose for the induction of transdifferentiation has been reached in animal models of the phenomenon, no further increase in effect has been observed. Additionally, no inhibitory effect was observed at higher doses, which suggests that the lack of human transdifferentiation in this instance is unlikely to be as a result of an inhibitory dose of bFGF.

The fact that this experiment has shown that nestin expression is induced in response to bFGF, would suggest that a down-regulation of FGFR-1 is not responsible for the loss in capacity for transdifferentiation, given that some, if not all of the signal has been transduced to the nucleus in order to elicit a transcriptional response. It is possible that bFGF is able to signal via another receptor, however, to our knowledge no other bFGF receptor has been reported. FGF-receptors are reported to require the binding of a co-factor, in this case heparin proteoglycan, in order to properly bind their soluble ligand (Yayon et al., 1991). The addition of heparin to the culture medium may therefore increase the effects of the exogenously added bFGF in the induction of transdifferentiation.

The lack of transdifferentiation in response to bFGF at this later stage of development, despite a limited response to the growth factor, may suggest that a crucial part of the bFGF signaling cascade is being attenuated. If this is the case then the identity of this mechanism is yet to be identified.

5.3 The potential for bFGF-induced transdifferentiation in primary human fetal RPE explants:

5.3.1 Introduction:

Human RPE tissue at a stage comparable to that of embryonic chick RPE which retains the capacity to undergo transdifferentiation is difficult to obtain in sufficient quantities for detailed investigation. The earliest available human RPE tissue had previously been from 8 week old embryos, which is developmentally more mature than the ideal stage for transdifferentiation when compared with chicken development (approximately HH33). This stage has been shown not to undergo transdifferentiation, however, human RPE from earlier stages, ideally 4-5 weeks, may exhibit the ability to undergo transdifferentiation if human cells behave in a similar manner to those of the embryonic chicken. However, this material is

very rare and therefore it is difficult to prove that human RPE cells are able to transdifferentiate like their animal counterparts. This is the principle reason that on-going studies in the chicken model are being undertaken, in order to better understand what it is that restricts the potential for transdifferentiation of the RPE towards neural retina. However, following the earlier investigation, human fetal RPE cells at an earlier stage of 6 weeks of age became available (CS18). If human cells are able to transdifferentiate in a similar manner to chicken RPE cells, then this stage would likely fall within a period when chicken RPE has been shown to display evidence for transdifferentiation (approximately HH27). This tissue was therefore investigated for the potential for transdifferentiation using the established culture system employed in previous experiments.

5.3.2 Methods & Materials:

5.3.2.1 Primary RPE isolation & Culture:

Human fetal eyes at several different developmental stages were obtained and isolated as described in chapter 2.2. Explanted RPE was subsequently cultured for 10 days in the standard, non-adherent, transdifferentiation, culture system, +/-bFGF (100ng/ml) in HESC medium as described previously (Chapter 2.3).

5.3.2.2 Immunohistochemistry and statistics:

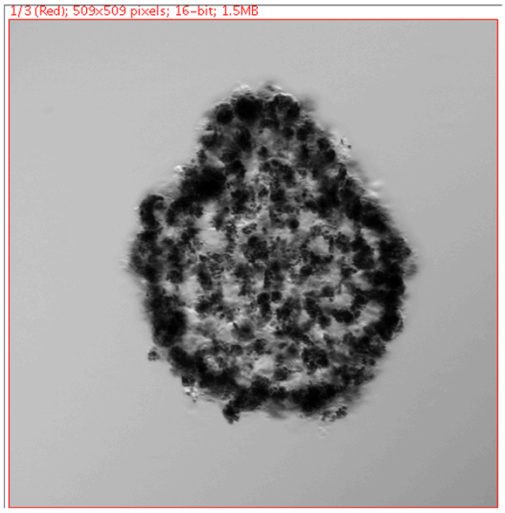
Immunohistochemical and statistical analysis was performed as described in chapter 2.4/2.11 respectively.

5.3.2.3 Image analysis:

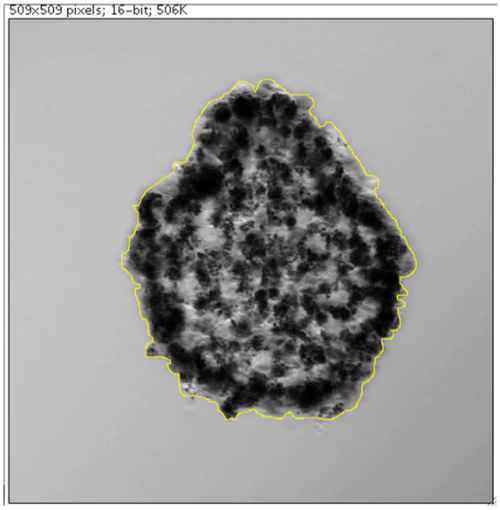
To quantify the levels of expression of RPE marker Pmel17, as well as the level of pigmentation in cultures of HESC-RPE and human fetal RPE cells, it was necessary to use an image analysis software, ImageJ. 16-bit grayscale images were produced as described previously (see previous experiment 5.3).

For cultures where multiple biological repeats were available [e.g. human fetal RPE (CS18, CS19)], it was possible to select the area of the whole section, using the *region of interest* tool (yellow line in Fig. 5.M3), for each image, for each biological repeat, and then average the average pixel intensity for each image for each culture condition. These averages for each cell type, in each culture condition, could therefore be compared graphically to analyse the effects of different cultures systems on the level of expression of Pmel17, and level of pigmentation. Additionally, for some explants, multiple nomarsky images were available for each biological repeat, and therefore, these were included in the average level of pigmentation calculation in order to increase the accuracy of the overall average. It was therefore possible to calculate the standard error for each average, which were represented as error bars.

In some instances, namely for the quantification of CS22 human fetal RPE explants, there was only 1 biological repeat available owing to the rarity of the tissue. In these circumstances in was more difficult to accurately quantify the Pmel17 expression, and pigmentation level because of the lack of repeats. In an effort to assess the variability of the values for these variables, several *regions of interest* for each image were calculated for a single image (See yellow lines Fig. 5.M4). These regional values were then averaged to give an overall average for each image, which allowed a standard error calculation for each image to be performed. In this instance, the standard error bars realistically reflect the variability in the levels of Pmel17 and pigmentation within a singular culture condition, rather than the likely error between multiple biological repeats.



Open Fluoresence/Nomasrky image in ImageJ.



Convert the image to a 16-bit grayscale image.

Use *region of interest* tool to select the whole section of RPE.

Results		
Mean	Min	Max
78.191	0	255

Measure average level of pixel intensity on the grayscale. 0 = Black, 255 = White.

Fig. 5.M3
Quantification of the average pixel value for a whole section using Imagej software.
Image analysis software was employed to quantify to average pixel intensity value of a given area, in this case, the whole section of an explant, selected using a region of interest tool (yellow line). The average pixel intensity of pixels in this slected region can then be calculated using the software, and is expressed on a grayscale. Black (0), White (255).

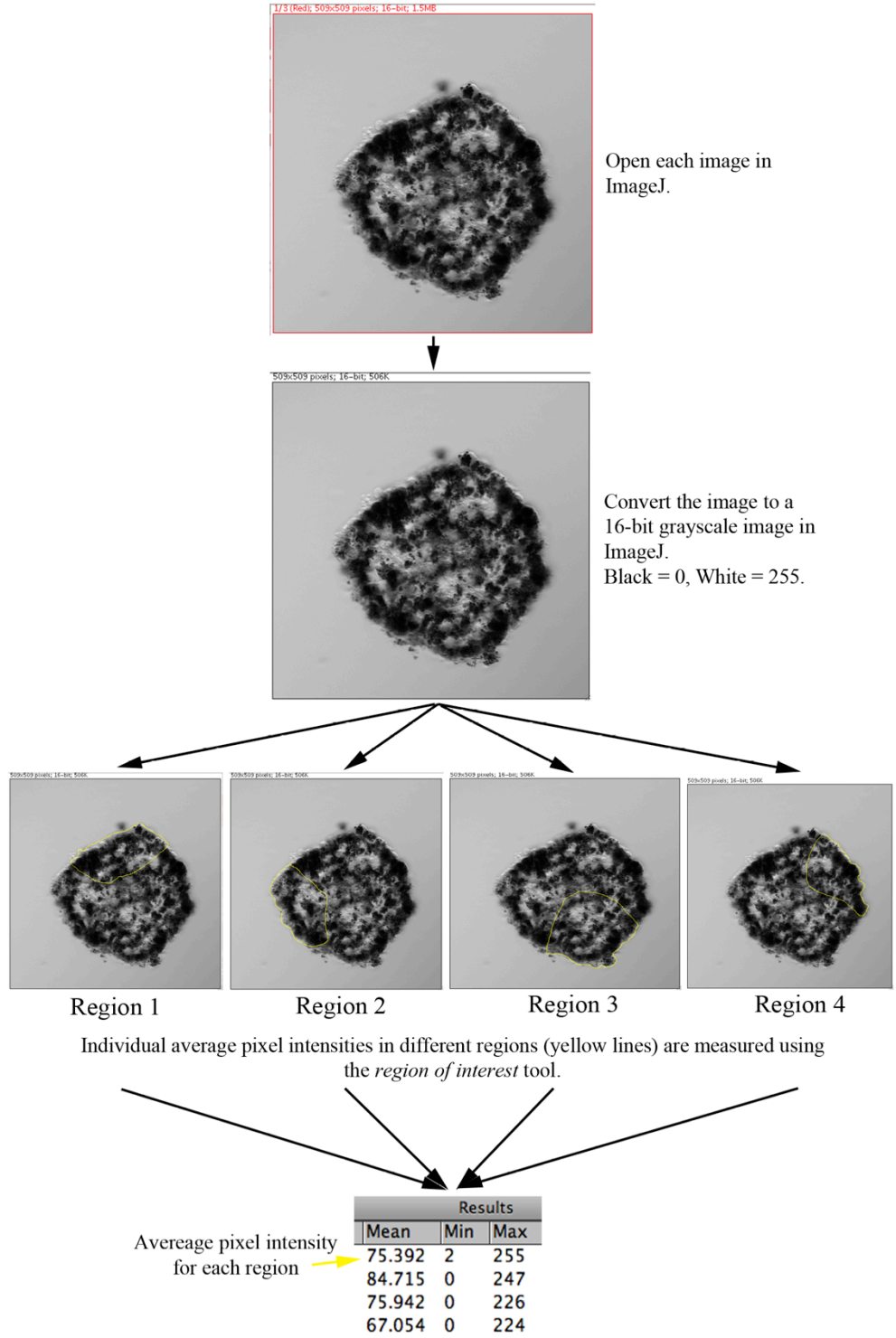


Fig. 5.M4
Quantification of the average pixel value of several regions of the same section using ImageJ software.
In order to calculate regional-specific expression levels of different markers/pigmentation, it was necessary to employ the use of image analysis software to firstly highlight the different regions of interest (yellow lines) on a grayscale image of each channel of interest. Subsequently, the software can then be used to calculate the average pixel intensity for all the pixels within the selected region. The average pixel intensity is expressed on a grayscale where Black = 0 and White = 255.

5.3.3 Results:

5.3.3.1 CS18 RPE:

The earliest available primary human fetal RPE tissue was aged approximately 6 weeks at CS18. Treatment of this tissue with 100ng/ml bFGF in the standard, non-adherent, transdifferentiation, culture system appeared to induce a level of de-pigmentation of the RPE tissue (Fig. 5.23 B, C, 5.8B, 5.9A) when compared to untreated negative controls (Fig. 5.23A, 5.8A, 5.9B). Additionally, areas of the bFGF treated explants appeared to be multi-layered and neuroepithelial in nature, as displayed by the nuclear labelling using DAPI (Fig. 5.23B, C, 5.24B, 5.25A) whereas the negative controls appear to have remained as a folded, single layer of cells (Fig. 5.23A, 5.24A, 5.25B). Discreet regions in both explants cultured with bFGF (n=2 where n = no. of biological repeats) displayed neuroepithial characteristics akin to an immature, developing retina (Fig. 5.23E, H, K, N, F, I, L, O white boxes; 5.24B, D, F, H, J; 5.25B, C, E, F). This region was multi-layered and expressed neural progenitor marker Sox2 across the neuroepithelial structure (Fig. 5.23E, F), however, Sox2 expression was absent in the untreated negative control (Fig. 5.23D). Sox2 expression in the bFGF treated explant was largely co-localized with that of nuclear Pax6 expression in the neuroepithelial structure. (Fig. 5.23H, I, N, O). Interestingly, Pax6 expression was present throughout the bFGF treated explant (Fig. 5.23I, O), however, the level of intensity of expression appeared to be lower in the rest of the explant (Fig. 5.23I), compared with the neuroepithelial structure (Fig. 5.23I white box). The other explant that appeared to display some level of transdifferentiation did not exhibit Pax6 expression outside of the neuroepithelial region. Similarly, untreated explants also displayed Pax6 expression within most cells of the cultured RPE (Fig. 5.23G, J, M), however, the level of expression appeared to be less than most of the cells expressing Pax6 in bFGF treated explants, including the regions which did not exhibit evidence of transdifferentiation (Fig. 5.23G-I). Pmel17 expression was very robust in untreated negative controls (Fig. 5.23J) as would be expected for heavily pigmented cells, which appear to have

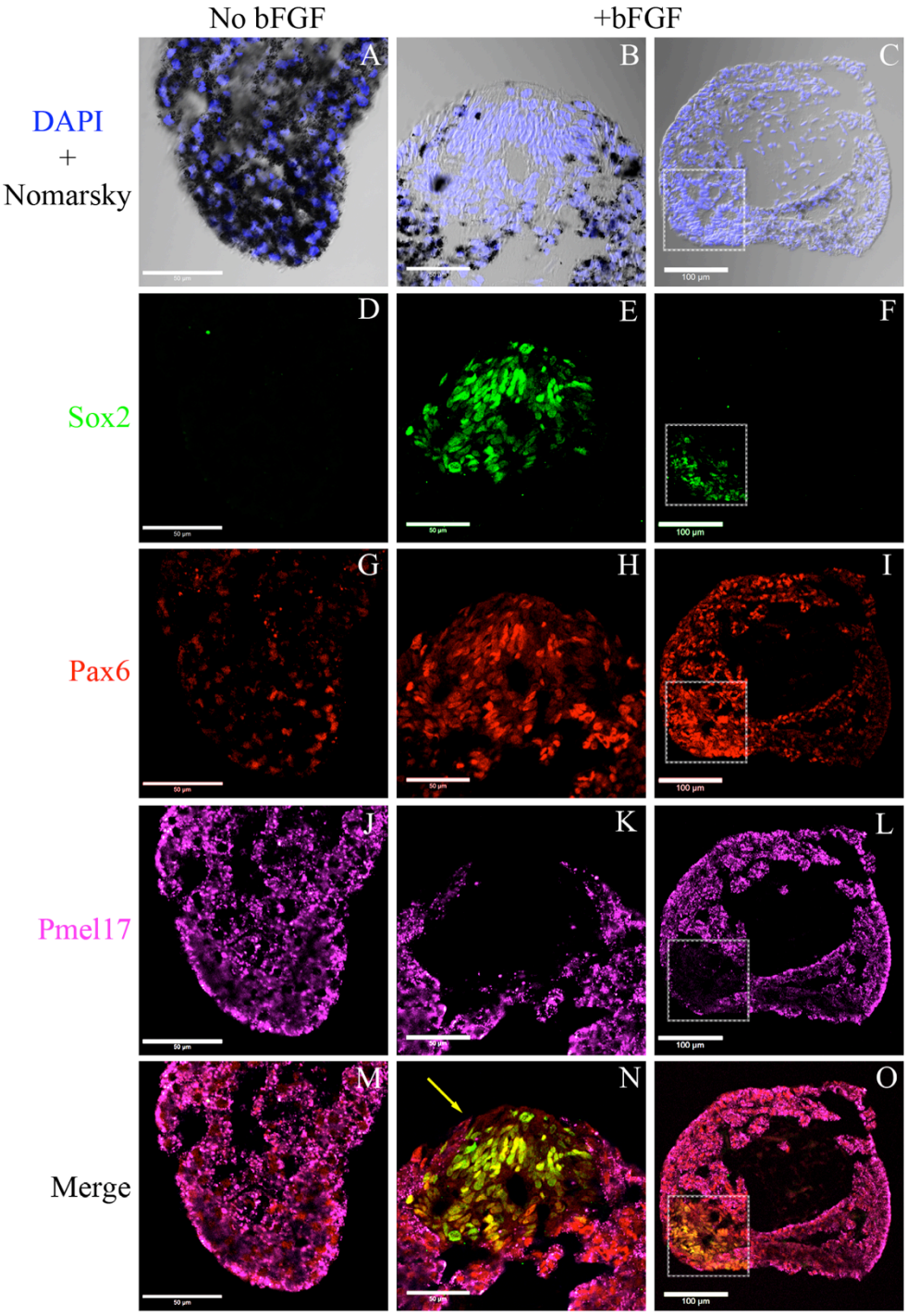


Fig. 5.23
Evidence for transdifferentiation of human fetal RPE explnats, CS18 towards a neuronal phenotype in response to bFGF after 10 days.
A region of human RPE explant treated with bFGF (100ng/ml) was observed to display a non-pigmented, neuroepithelial phenotype which was continuous with Pmel17 positive, pigmented RPE cells (C, F, I, L, O white box, High mag. (B, E, H, K, N yellow arrow). This region expressed markers consistent with developing neuroretinal progenitors Pax6 (H, I), and Sox2 (E, F), which also co-localised in this region. Sox2 was absent from Pmel17 positive RPE cells in the bFGF treated explant (F), as well as the entirity of the untreated explant (D). No neuroepithelial regions were observed in the untreated (-bFGF) negative control, which retained a pigmneted, Pmel17 positive (J) RPE phenotype. However, low Pax6 expression was detected in these cells in one explant (G). Pax6 expression was also observed throughout the bFGF treated explant, including areas which retained the RPE phenotype (I, O). bFGF treated RPE appeared less pigmneted than untreated explants after 10 days in culture (A-C). Scale bars: 100uM.

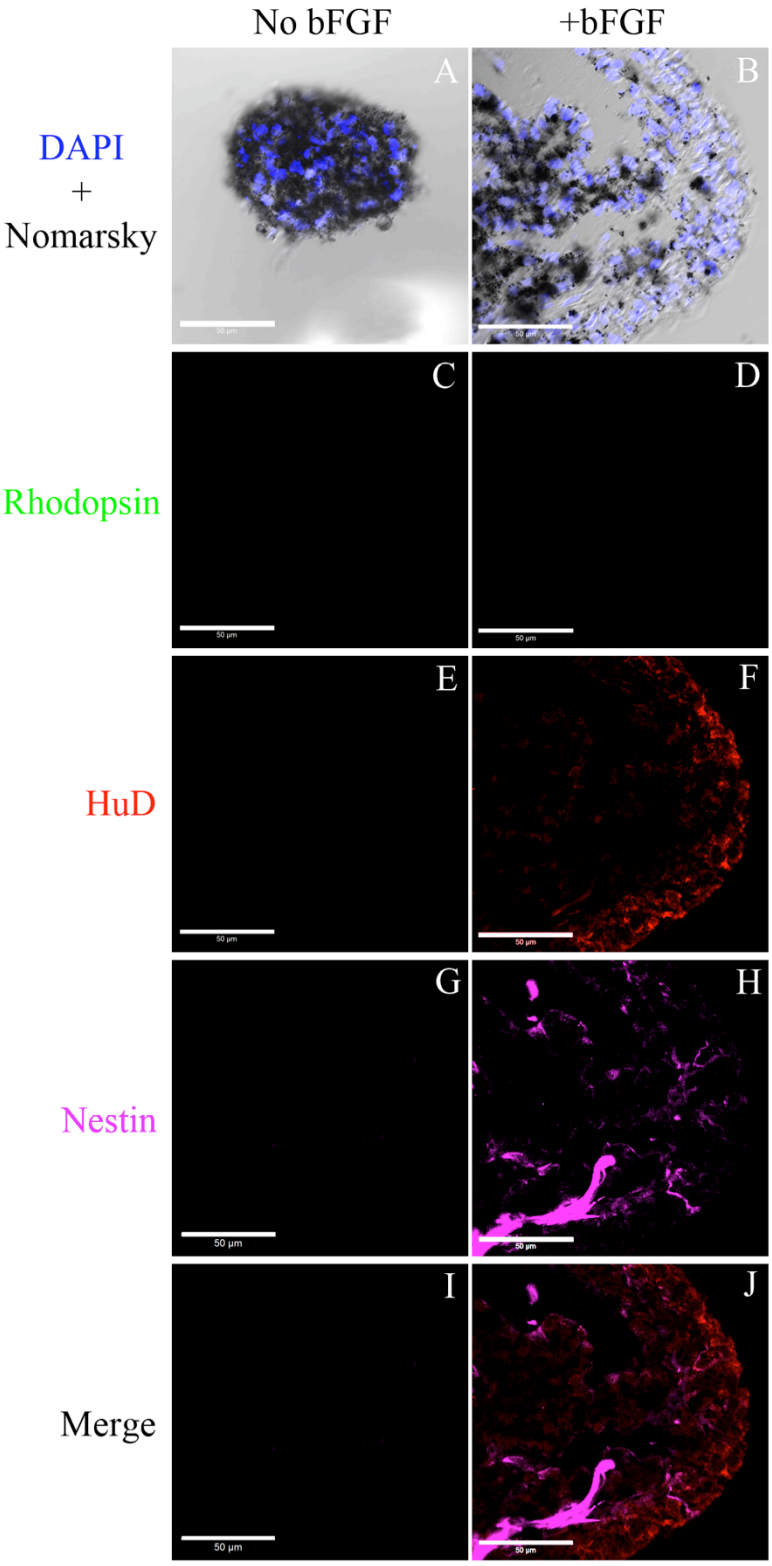


Fig. 5.24
Evidence for transdifferentiation towards a neuroretinal phenotype in human fetal RPE explants, CS18 after treatment with bFGF for 10 days.
RPE treated with bFGF was observed to exhibit less pigmentation than the -bFGF negative control (A, B). HuD expression was exhibited in some cells throughout a somewhat neuroepithelial region in the bFGF treated explant (F) which was absent from the untreated control (E). Similarly, many of these cells also expressed neuronal progenitor marker, nestin (H, J). No nestin was expressed in the negative control (G). Rhodopsin expression was absent from both explants (C, D). Scale bars: 50uM.

retained their characteristic RPE phenotype. Pigmentation was observed to co-localise with Pmel17 expression, supporting the idea that an RPE phenotype has been retained (Fig. 5.23M). bFGF treated explants also robustly expressed Pmel17 throughout, however, the region which showed evidence of potential transdifferentiation was largely negative for Pmel17 expression (Fig. 5.23L, K). This is characteristic of cells that have lost their pigmented RPE phenotype, and combined with the expression of immature neural retinal markers (Fig. 5.23N yellow arrow), suggests a re-specification as retinal tissue. Additionally, the apparently transdifferentiated region of the bFGF treated explant appeared to be continuous with the Pmel17 positive, pigmented RPE cells which have retained their characteristic phenotype (Fig. 5.23N), which supports the idea that this neuroepithelial region has resulted from re-differentiation of the cultured RPE cells. bFGF treated explants were also observed to express another neuronal marker associated with RPE to neural retina transdifferentiation, HuD (Fig. 5.24F, 5.25C). The protein was expressed throughout a lightly pigmented region of one explant (Fig. 5.24B), and in a completely de-pigmented region of another (Fig. 5.25C), which were apparently multi-layered in structure, in a similar manner to a neuroepithelium (Fig. 5.24B, 5.25A). HuD was localised to the cytoplasm of these cells as expected (Fig. 5.24F, 5.25C) and was observed throughout the less pigmented region of one explant (Fig. 5.24B, F), but in only a few cells of the non-pigmented region of another (Fig. 5.25C). The untreated negative controls were negative for the expression of HuD (Fig. 5.24E, 5.25F). Interestingly, nestin expression was observed throughout the bFGF treated explant (Fig. 5.24H) and in some cases co-localised with the expression of HuD in the less pigmented region (Fig. 5.24J). The nestin expression appeared in a fibrillar pattern that spanned the breadth of the less pigmented region in bFGF treated explants (Fig. 5.24H, J) and was perpendicular to the neuroepithelial-like structure. Again, the neuronal marker nestin was absent from untreated negative controls (Fig. 5.24G, I). Despite the apparent transdifferentiation of RPE cells towards neural retinal cells in bFGF treated CS18 human fetal RPE explants, no rhodopsin expression was observed in either bFGF treated or untreated explants (Fig. 5.24 C, D, 5.25B, E).

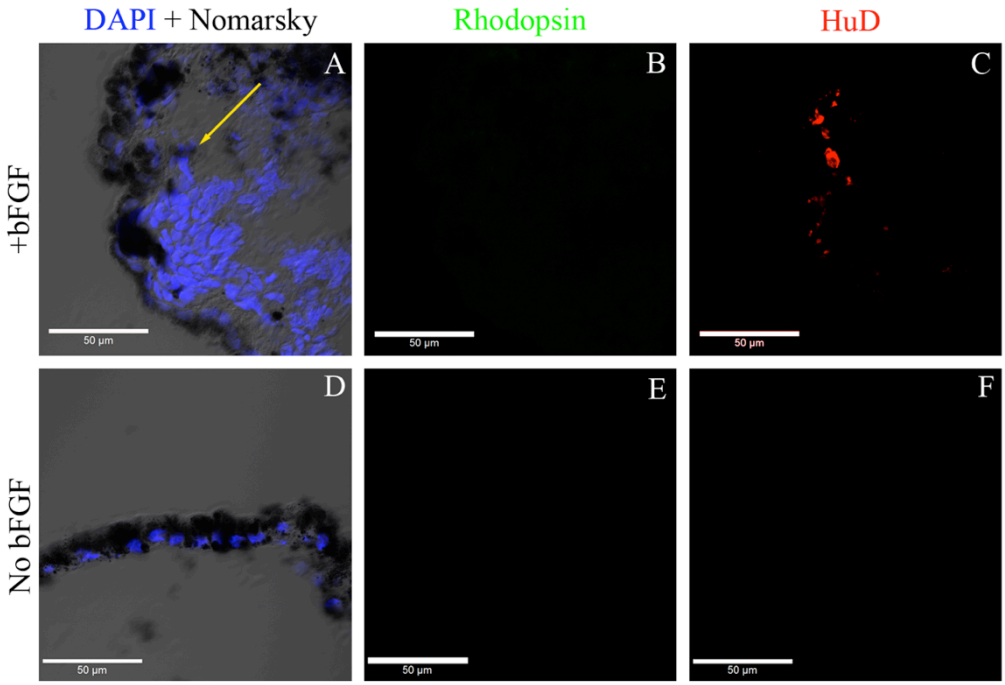


Fig. 5.25
Evidence for transdifferentiaiton in human fetal RPE explants, CS18, treated with bFGF (100ng/ml) for 10 days - embryo 2.
A de-pigmented, neuroepithelial-like region of multiple layers of cells was observed in the bFGF treated explant (A, yellow arrow), whereas no such structure was observed in the negative control, which retained an RPE monolayer phenotype throughout the explant (D). The neuroepithelium did exhibit some HuD expression which was continuous with pigmented RPE cells (C) but no HuD was present in the negative control (F). No rhodopsin expression was observed in either explant (B, E). Scale bars: 5ouM.

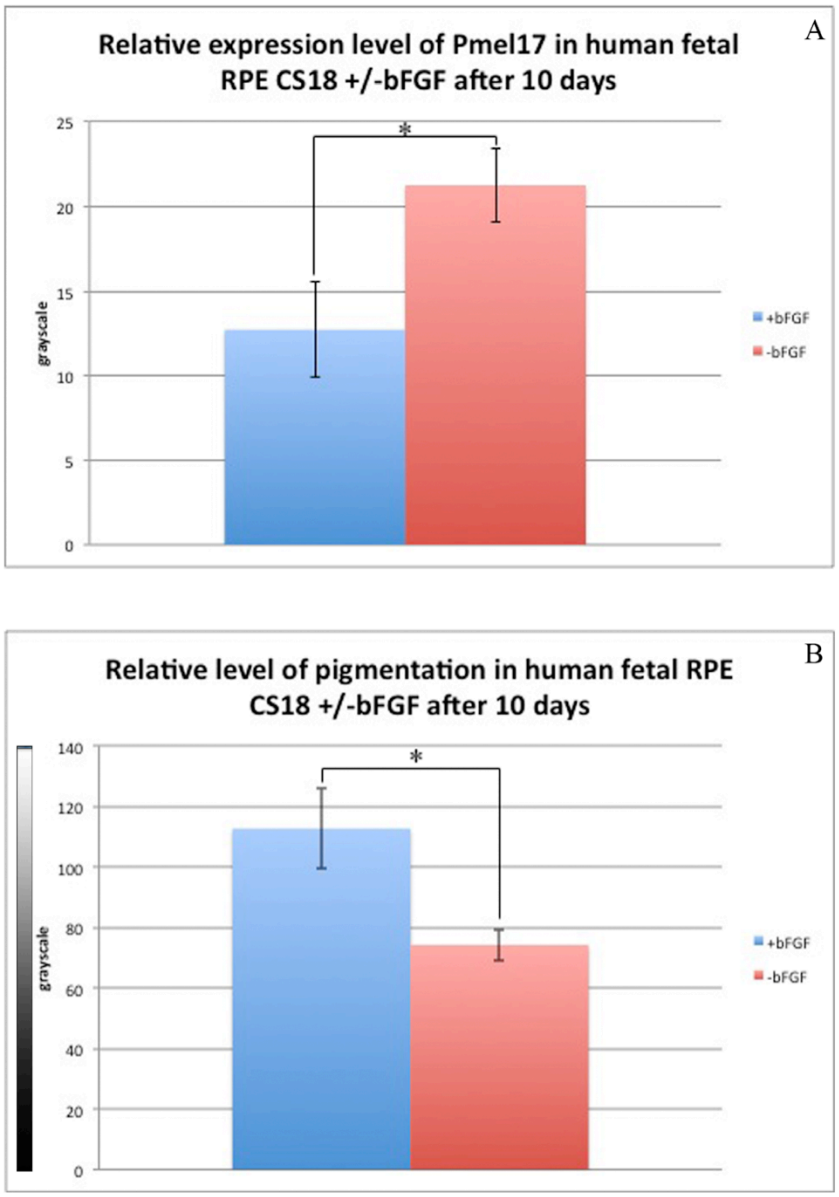


Fig. 5.26

The quantification of the relative level of expression of Pmel17 (A) and pigmentation (B) in human fetal RPE explants, CS18, +/-bFGF(100ng/ml) after 10 days.

Image analysis software was used to measure the average pixel intensity of each whole section for each explant. (A) Pmel17 expression was observed to be significantly lower in bFGF treated explants compared with untreated, negative controls ($p<0.05$, RANOVA, $n = 2$) suggesting a move away from an RPE phenotype. (B) bFGF treated RPE explants were significantly less pigmented than untreated negative controls ($p<0.05$, RANOVA, $n = 2$). Grayscale: Black = 0, White = 255. Error bars: Standard error.

Given the fact that the bFGF CS18 human fetal RPE explants appear to have less pigmentation than the untreated negative controls, image analysis was employed in order to attempt to quantify the relative level of pigmentation in explants treated both with and without bFGF. It was found that the average pixel intensity of bFGF treated explants was higher than that of untreated negative controls (Fig. 5.26B) and that this difference was statistically significant ($p<0.05$, $n = 9$, where n is the number of different images measured from 2 biological repeats, RANOVA). A significantly higher average pixel intensity implies that bFGF treated explants are lighter in appearance than untreated negative controls, which are therefore more pigmented. Interestingly, the lighter bFGF treated explants also correlated with a significant loss in the level of expression of Pmel17 compared with the untreated negative controls, and this difference was found to be statistically significant ($p<0.05$, $n = 5$, where n is the number of different images measured from 2 biological repeats, RANOVA).

5.3.3.2 CS19 RPE:

Generally speaking, CS19 human fetal RPE explants (n=2) did not express markers of transdifferentiation in response to bFGF treatment (Fig. 5.27, 5.28). One bFGF treated explant did not appear to lose pigmentation in response to the growth factor treatment (Fig. 5.27A, K) when compared with the untreated, negative control (Fig. 5.27F, P). Both bFGF treated and untreated explants retained the expression of RPE marker, Pme17, which is consistent with the retention in pigmentation of the explants (Fig. 5.27E, J). The level of expression of Pmel17 appeared to be comparable between bFGF treated explants and untreated explants. No Sox2 expression was observed in bFGF treated, or untreated explants (Fig. 5.27C, H), and the same was true for Pax6 expression, which was negative in both culture conditions (Fig. 5.27D, I). As expected for explants, which did not display other characteristics of transdifferentiation in response to bFGF, rhodopsin expression was negative in both culture conditions (Fig. 5.27M, R), as was the neural retina marker, HuD (Fig. 5.27N, S). Despite the fact that no evidence of RPE to neuronal transdifferentiation was apparent in

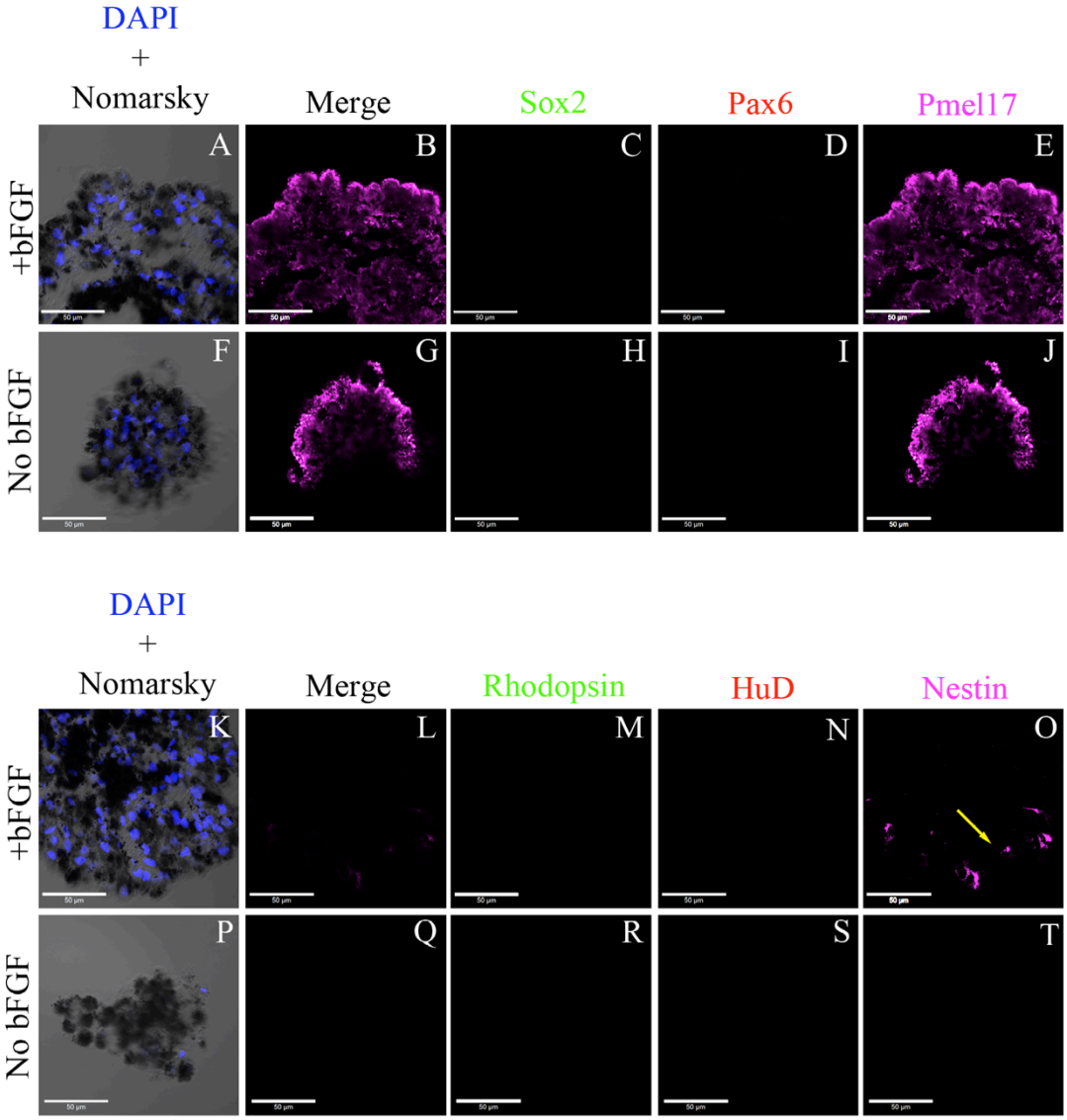


Fig. 5.27
Human fetal RPE explant, CS19, +/-bFGF (100ng/ml) for 10 days - Explant 1.
bFGF treated RPE cells appeared to maintain the characteristic pigmentation (A, K) and Pmel17 expression (E, O) comparable to the untreated control (F, P, J, T). Retinal progenitor markers, Pax6 (D, I) and Sox2 (C, H) were absent from both +/-bFGF treated cultures which is consitent with no transdifferentiation. Consistent with this, HuD (N, S) and Rhodopsin (M, R) were also absent from cultures. Nestin expression was observed at low levels in some bFGF treated RPE (O, yellow arrow) but not in untreated cultures (T), which suggests that despite the lack of transdifferentiation, CS19 RPE can still respond to bFGF at some level to initiate neuronal gene expression. Scale bars: 50uM.

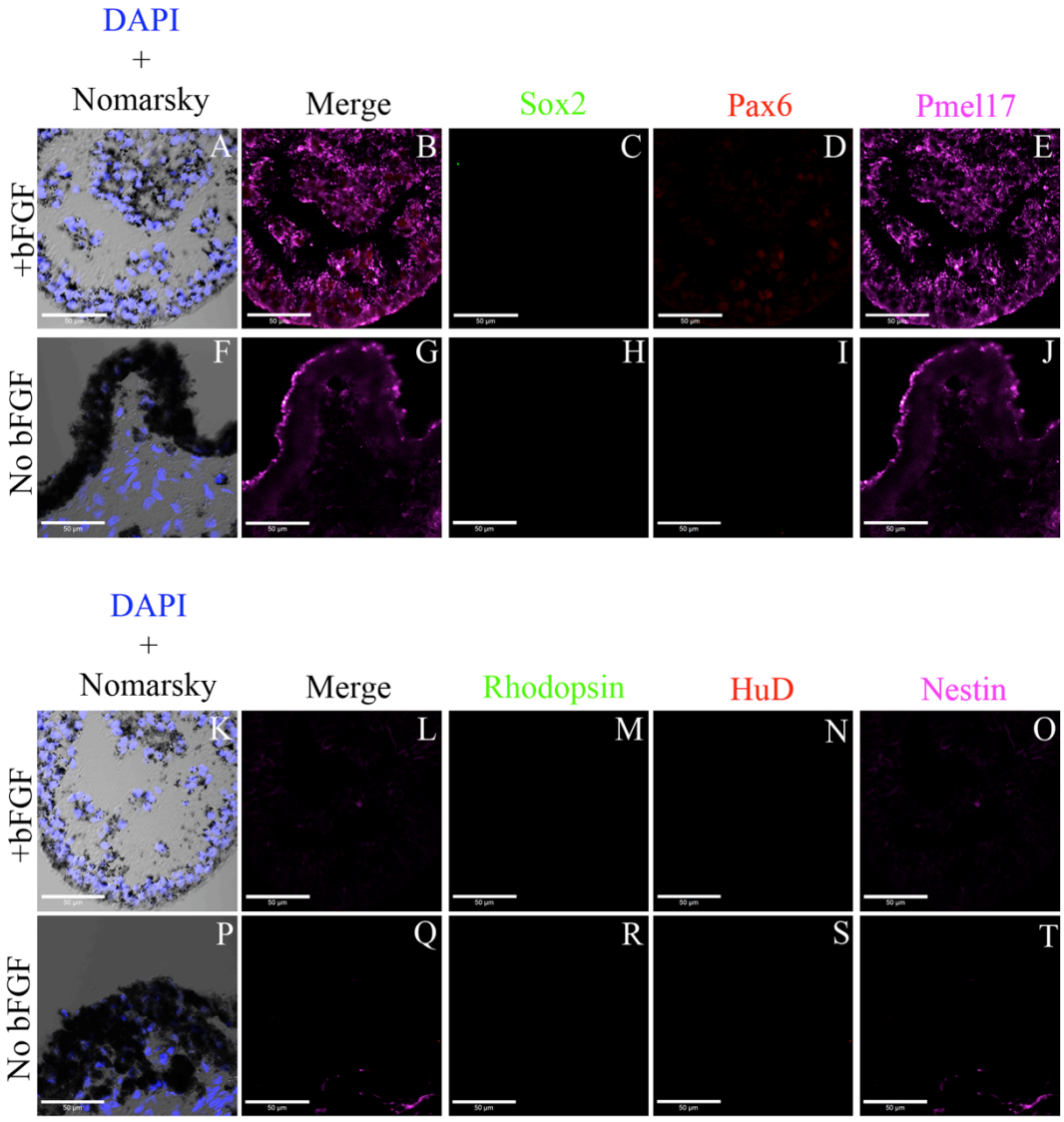


Fig. 5.28
Human fetal RPE CS19 explants +/-bFGF for 10 days - Explant 2.
This human fetal RPE explant treated with bFGF appeared to be less pigmented (A, K) than the untreated negative control (F, P). Additionally, bFGF treated RPE exhibited thickened regions which appeared multi-layered (A, K) when compared with untreated RPE (F, P). Both treated and untreated explants retained RPE marker, Pmel17 expression (E, J) and did not express neuronal progentior marker Sox2 (C, H), or differentiated retinal cell markers HuD (N, S) or rhodopsin (M, R). This would suggest that transdifferentiation hasn't taken place. However, weak Pax6 expression was observed in some bFGF treated RPE cells (D) but not in unrteated cells (I). Nestin labelling was barely detectable in both +/- bFGF cultures in these sections (O, T). Scale bars: 50uM.

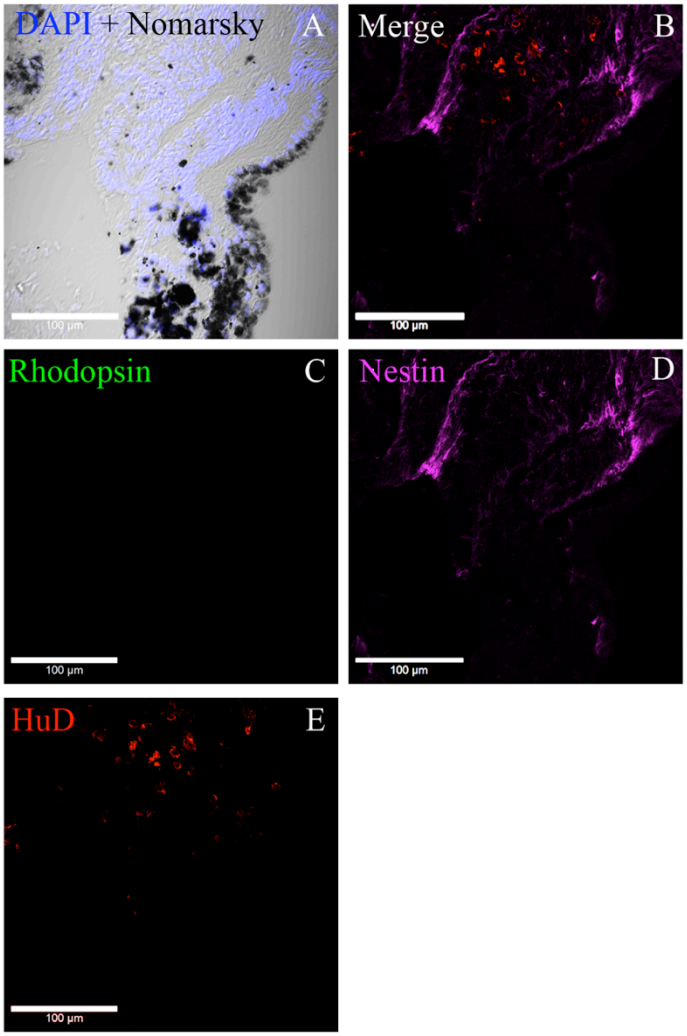


Fig. 5.29
Evidence for transdifferentiation of CS19 RPE +bFGF for 10 days - Explant 2, different section.
Given the fact that explant 2 displayed de-pigmentation in response to bFGF, another section from the same explant exhibiting de-pigmented regions was investigated for transdifferentiation. This section displayed large non-pigmented, neuroepithelial regions, which were continuous with heavily pigmneted RPE cells (A). Some pigment granules were still present in these structures which may suugest previous specification as RPE cells (A). Neuroepithelial structures exhibited both nestin (D) and HuD (E) expression, which was absent from adjacent pigmented regions. No rhodopsin expression was present (C). The above evidence would suggest that transdifferentiation has taken place. Scale bars: 100uM.

this explant, some cells were observed to exhibit low levels of nestin expression in response to bFGF (Fig. 5.27O, yellow arrow), which was absent from the untreated negative control (Fig. 5.27T). However, another CS19, human fetal RPE explant was observed to display other responses to bFGF treatment following the 10 day culture period. The bFGF treated RPE was visibly lighter in appearance (Fig. 5.28A, K) than the untreated RPE control (Fig. 5.28F, P). Additionally, the bFGF treated explant appeared to display regions where cells were multi-layered as observed using a DAPI, nuclear stain (Fig. 5.28A, K) in comparison with the untreated controls which exhibited a characteristic, RPE monolayer organisation (Fig. 5.28F, P). The pigmentation in bFGF treated cells was noticeably more granular and sparsely spread throughout the explant (Fig. 5.28A, K), whereas untreated controls appeared much more uniform and intense (Fig. 5.28F, P). Despite this difference, both bFGF treated and untreated explants were observed to express relatively comparable levels of Pmel17 (Fig. 5.28E, J), as with the explants from a separate eye. In keeping with retention of the RPE phenotype, no Sox2 expression was observed in either culture condition (Fig. 5.28C, H), and the same was the case for both Rhodopsin (Fig. 5.28M, R), and HuD (Fig 5.28N, S).

Interestingly, in this explant, the RPE treated with bFGF did appear to express a low level of Pax6 in the nuclei of some cells (Fig. 5.28D), which was absent in the untreated control (Fig. 5.28I). Similarly, a very low level of nestin expression was present in the bFGF treated explant (Fig. 5.28O), which was not present in the untreated negative control (Fig. 5.28T). Further investigation of another CS19 RPE section +bFGF from the same culture (same eye) was investigated for the expression of retinal markers, given the apparent de-pigmentation in response to bFGF discussed earlier. An area of largely non-pigmented, bFGF treated explant was observed to contain what appears to be a multi-layered, neuroepithelial structure, which is continuous with the pigmented RPE regions of the explant (Fig. 5.29A), as confirmed via DAPI labelling. Areas of this de-pigmented region displayed nestin positive fibres, which were also present in some of the pigmented cells of the explant, in response to bFGF (Fig. 5.29B, D). Additionally, HuD expression was present in the cytoplasm of a number of cells in the region of the de-pigmented, apparently neuroepithelial, structure

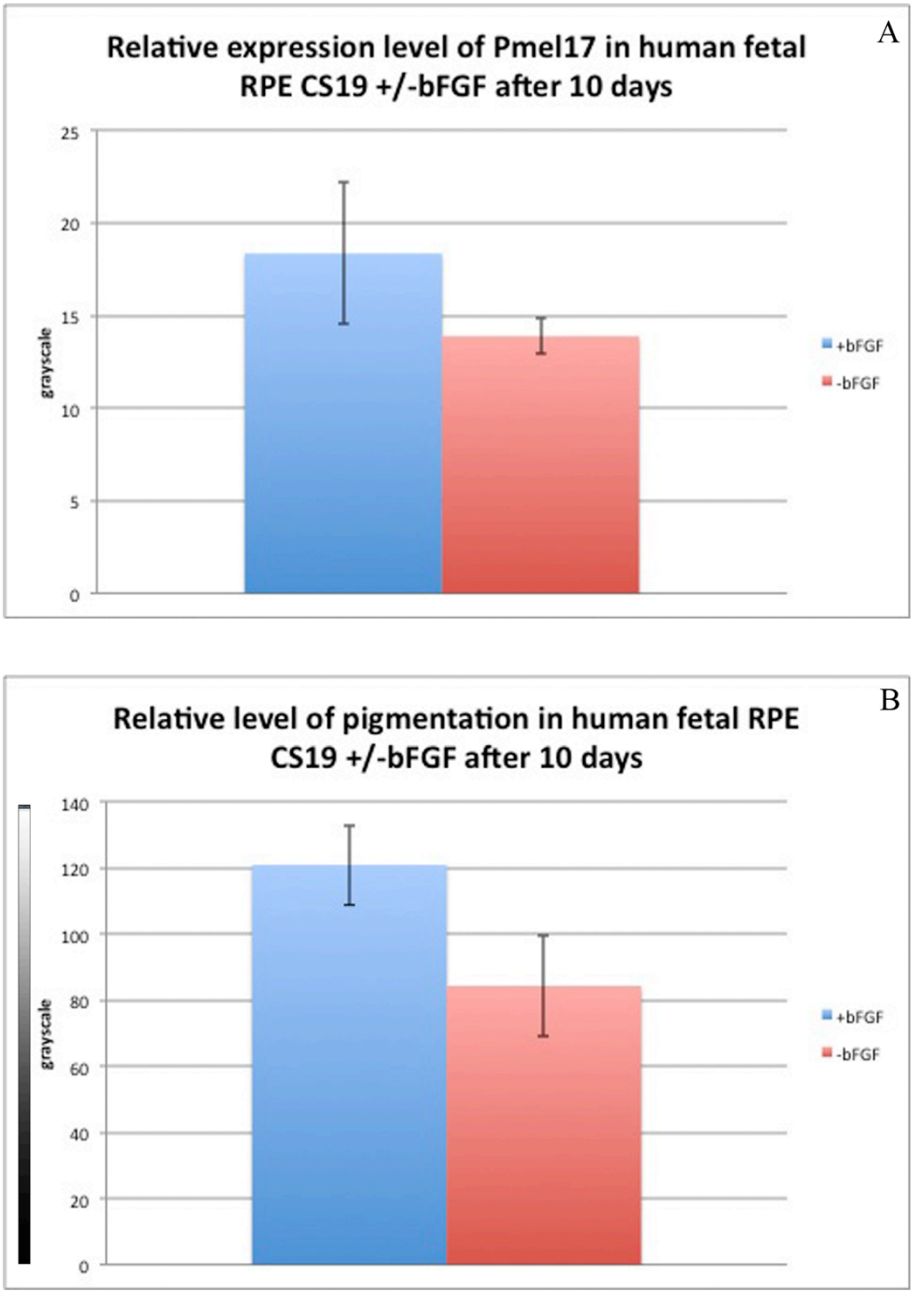


Fig. 5.30
Quantification of the expression level of Pmel17 and pigmentation in Human fetal RPE CS19 explants +/-bFGF for 10 days, using image analysis software.
No significant difference in the level of Pmel17 expression was observed between bFGF treated, and untreated explants (A). bFGF treated explants were found to be significantly less pigmented than untreated controls (B), however, this difference was not statistically significant. Error bars: standard error.

(Fig. 5.29E). However, no cells in this section were observed to express rhodopsin (Fig. 5.29C). Image analysis of all these sections was undertaken in order to ascertain whether or not the bFGF was able to have an effect on the level of pigmentation, as well as the level of expression of Pmel17, in CS19 RPE explants. Quantification of the level of expression of Pmel17 expression was not found to be significantly different between bFGF treated explants, and untreated controls (Fig. 5.30A). However, bFGF treated explants were found to be less pigmented than their untreated counterparts, however, this difference was not found to be statistically significant (Fig. 5.30B).

5.3.3.3 CS22 RPE:

CS22 human fetal RPE explants treated with and without bFGF did not display any evidence of transdifferentiation towards a neural retinal phenotype (Fig. 5.31)(n=1). bFGF treated and untreated negative control explants displayed comparable levels of pigmentation (Fig. 5.31K, P), and both retained the characteristic Pmel17 expression maintained in RPE cells (Fig. 5.31E, J). Developing neural retina markers were absent from the pigmented explants in both culture conditions after 10 days. This included the absence of Sox2 expression (Fig. 5.31C, H), HuD expression (Fig. 5.31N, S), and rhodopsin expression (Fig. 5.31M, R). Interestingly, no Pax6 expression was present in either bFGF treated or untreated RPE explants (Fig. 5.31D, I), unlike RPE treated with bFGF at earlier stages. Only a very weak signal for nestin labelling could be detected in bFGF treated RPE (Fig. 5.31O), which was absent from the negative control (Fig. 5.31T), however, this signal was difficult to distinguish from the background signal, and may be a false positive. Despite the level of pigmentation appearing comparable in both bFGF treated, and untreated explants, image analysis software used to measure the average pixel intensity of each whole image suggested that Pmel17 expression was higher in bFGF treated RPE than untreated RPE (Fig. 5.32A). Similarly, bFGF treated RPE was observed to be lighter than the untreated control (error bar is standard error calculated using multiple images of the same explant) (Fig. 5.32B).

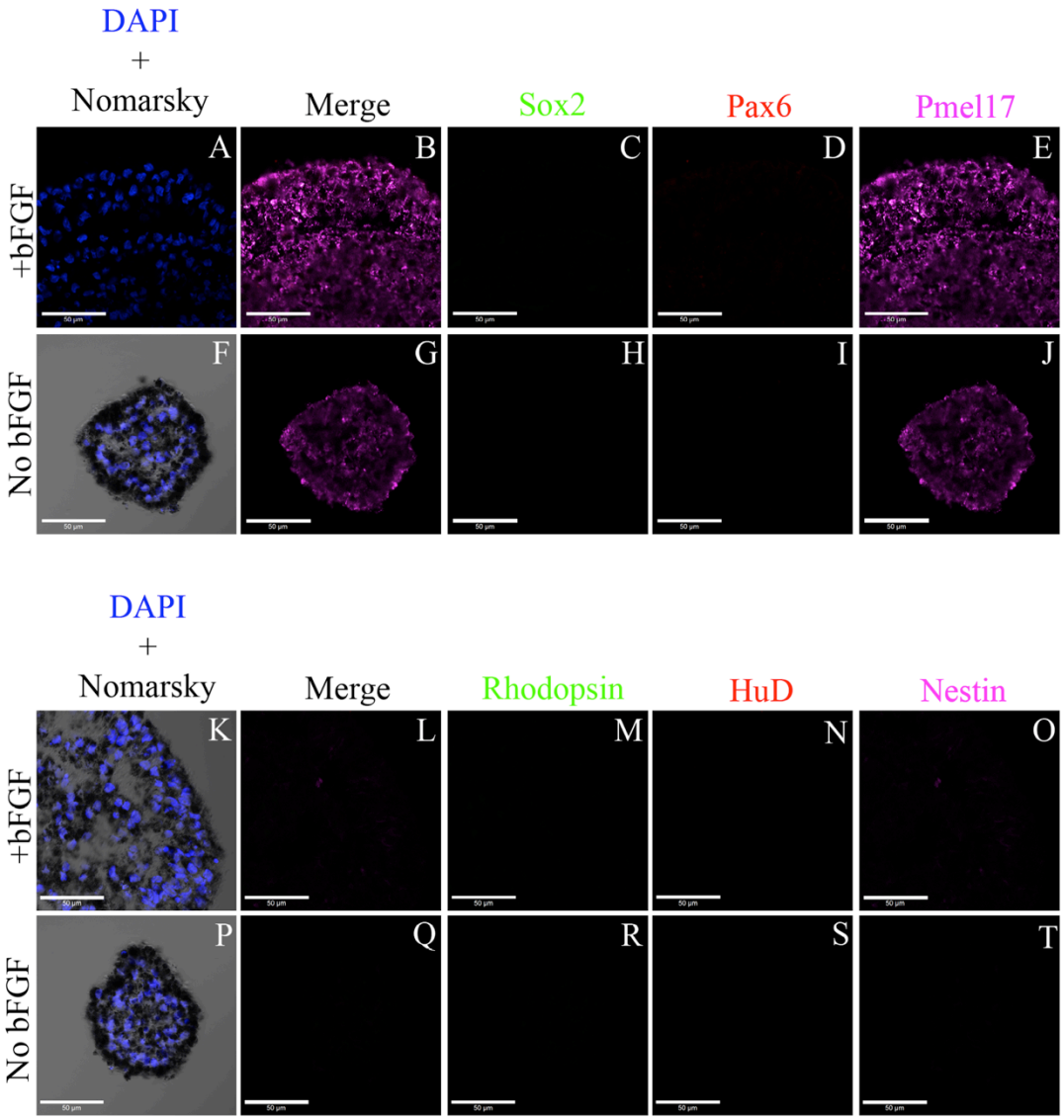


Fig. 5.31
Human fetal RPE CS22 explant treated with +/-bFGF (100ng/ml) for 10 days.
bFGF treated RPE did not appear to display a significantly less pigmentation (K) than untreated RPE (F, P). Both bFGF treated, and untreated RPE cells were observed to express Pmel17, which is characteristic of a retention of the RPE phenotype. Consistent with this, no retinal markers, Sox2 (C, H) , Pax6 (D, I), HuD (N, S) or rhodopsin (M, R) were expressed in either culture condition. However, very weak nestin expression was observed in a couple of cells of bFGF treated RPE (O), which was absent from untreated cells (T), however, it is possible that this signal is background. Therefore, depsite possible induction of neural marker expression, no robust transdifferentiation was observed to take place. Scale bars: 50uM.

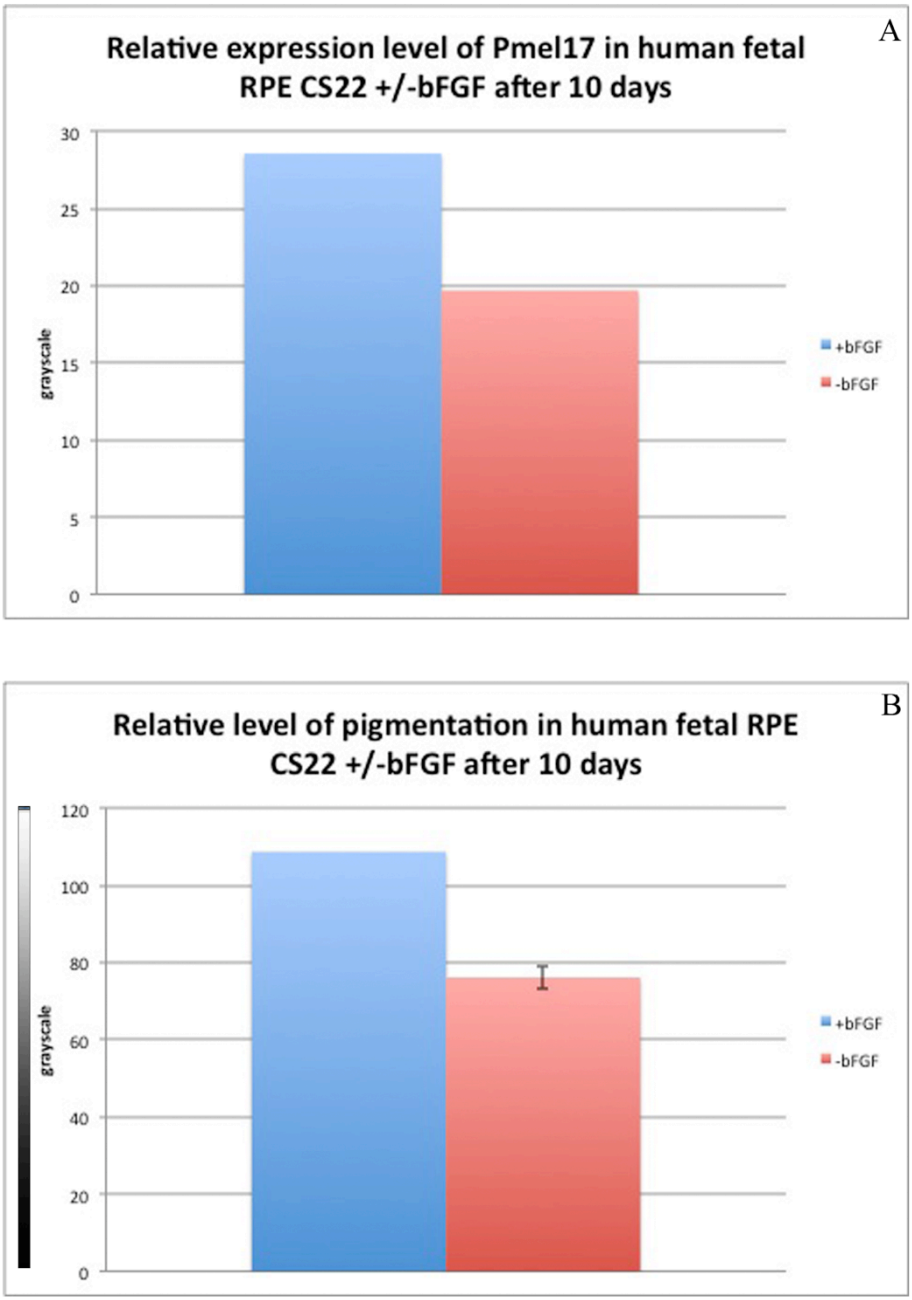


Fig. 5.32
Quantification of the expression levels of Pmel17 and pigmentation in human fetal RPE explants, CS22, +/-bFGF (100ng/ml) for 10 days, using image analysis software.
It is difficult to accurately quantify the expression of whole section images when only 1 biological repeat owing to the difficulty in estimating the error through calculation of standard error. (A) It appears as though Pmel17 expression is higher in bFGF treated cells, however, the lack of repeats means it is not possible to ascertain the error involved in this quantification, and therefore, this trend is not reliable. (B) Similarly, bFGF treated cells appear less pigmented than untreated cells as might be expected, however, the lack of repeats means that the reliability of this observation is in doubt. Error bars: Standard error. Grayscale: 0 = black, 255 = white.

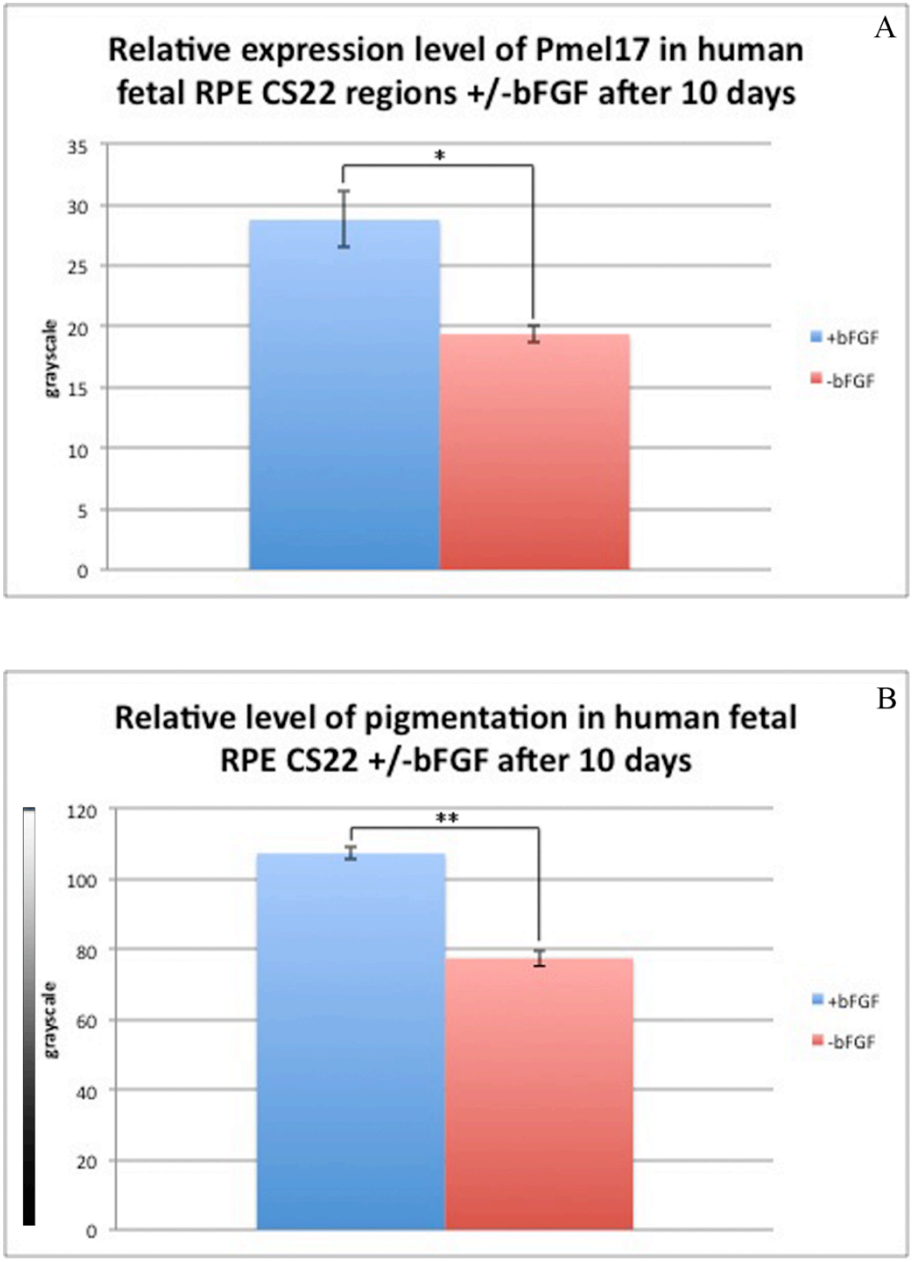


Fig. 5.33
Quantification of the regional level of expression of Pmel17 and pigmentation in human fetal RPE explants, CS22, +/-bFGF for 10 days, using image analysis software.
In order to attempt to account for the lack of biological repeats available for analysis, several different measurements were taken using the region of interest tool, for each image. (A) Pmel17 expression was observed to be significantly higher in bFGF treated RPE compared with untreated RPE ($p<0.05$, n = 4, where n = number of regional measurements, RANOVA). (B) bFGF treated RPE was significantly less pigmented than untreated RPE ($p<0.01$, n = 4, where n = number of regional measurements, RANOVA). Error bars: Standard error. Grayscale: 0 = Black, 255 = White.

However, the fact that only 1 biological repeat was available for quantification meant that it was difficult to calculate the error for most of the explants. Therefore, several regional measurements of the same image were taken in order to try to get a better idea of the variability of the level of expression of Pmel17, and the level of pigmentation, in RPE explants. Regional analysis suggested that Pmel17 expression in bFGF treated RPE was higher than in untreated negative control RPE, and that this difference is statistically significant ($p<0.05$, $n=4$, where n is the number of regions, RANOVA) (Fig. 5.33). Similarly, bFGF treated RPE was observed to be less pigmented than untreated negative control RPE cultures, and that this difference was also statistically significant ($p<0.01$, $n=8$, where 8 is the number of regional measurements, RANOVA). A summary of the numbers of explants that exhibited transdifferentiation is displayed in Table 5.2.

Developmental stage	Number of repeats (n)	Number of explants exhibiting transdiffernetiation
CS18	2	2
CS19	2	1
CS22	1	0

Table 5.2
A summary of the number of human fetal RPE explants of different developmental stages which were observed to undergo transdifferentiation in response to bFGF after 10 days in culture.

5.3.4 Discussion:

It appears that human fetal RPE cells can exhibit evidence of transdifferentiation in response to bFGF, in a similar manner to that of animal models, at early stages of development. This is the first time classical transdifferentiation of human RPE cells towards a neuro-retinal phenotype has been reported. Transdifferentiated RPE are observed to express expected markers of the developing neural retina, Pax6 and Sox2, however, only a few cells were observed to be HuD positive. This may suggest that the apparent transdifferentiation of human fetal RPE observed after 10 days in culture progresses at a slower rate than in the chicken model of transdifferentiation. This is to be expected given that the human gestation period is much longer than that of the chicken and therefore, eye development takes much longer. It is possible that longer culture of transdifferentiating human retina would yield the expression of more differentiated cell types, including differentiated retinal cell markers, such as rhodopsin, which was not observed in human fetal RPE cultures.

Evidence for transdifferentiation of human RPE in response to bFGF was only observed at the earliest stages of development of the RPE, at CS18/19, which is to be expected given parallels with animal models of the phenomenon (Coulombre, 1981, Coulombre and Coulombre, 1965, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993, Pittack et al., 1997, Pittack et al., 1991, Sakami et al., 2008). Further experiments using more human fetal tissue will be required in order to confirm this hypothesis, however, early indications that the capacity for bFGF becomes restricted in later stage RPE are encouraging for this hypothesis.

5.3.4.1 CS18 RPE:

Nevertheless, both CS18 explants treated with bFGF were observed to express retinal markers Pax6, Sox2, nestin and HuD within a largely non-pigmented, thickened neuroepithelial-like region, as would be expected from transdifferentiated human RPE cells if

they were to reflect transdifferentiation observed in animal models. These apparently transdifferentiated regions were somewhat similar in appearance and marker expression to transdifferentiated regions of embryonic chick RPE cells from cells isolated at later developmental stages (see chapter 5). Pax6 and Sox2 expression in the nuclei of apparently transdifferentiated regions co-localised in many cells, suggesting a neural retina progenitor identity. HuD expression was not observed to localize to the surface of a particular side of the neuroepithelial structure as one might expect for a marker of a differentiating retina, instead being located throughout the thickened region, however, this is can often be observed in transdifferentiated chick RPE, particularly given the difficulty in obtaining clear cross-sections from such small, disorganized tissue structures following culture. Therefore, HuD expression throughout the apparent neuro-retinal region does not necessarily rule out the presence of transdifferentiated retina. Given the likelihood that human retinal development would be much slower than its chicken counterpart, it is likely that any retina formed after only 10 days would be very immature. HuD expression may have only just initiated as ganglion cells and amacrine cells begin to differentiate. It may be that a longer developmental culture period is required for HuD expressing cells to migrate to the vitreal surface, where they are found in the mature retina. Nestin expression was observed to be characteristically fibrillar in nature in the de-pigmented regions, and perpendicularly arranged with regard to the length of the thickened region, as would be expected from developing neural retinal cells. Additionally, the thickened, retina-like, region appeared to be continuous with pigmented, Pmel17 positive RPE cells, which is also characteristic of transdifferentiated retina. Retinal markers were absent from heavily pigmented RPE cells in the explants as expected. This was also the case for untreated negative controls, except in the case of Pax6 expression for one CS18 explant, which was observed to express Pax6 throughout both bFGF treated, and untreated explants. Given the lack of Pax6 expression detected in the RPE of equivalent stage human fetal eye tissue at CS18, this could suggest that the non-adherent culture system is responsible for the expression of Pax6 in both treated and untreated explants, and is independent of exogenous growth factor treatment. However, the fact that Pax6 expression

was not detected in RPE cultures from another CS18 embryo implies that this is not necessarily the case. It is possible that Pax6 expression is maintained in some cells of the CS18 RPE monolayer, and not in others, as previously described for chick RPE monolayers, which down-regulated Pax6 in different regions with on-going development. This would thus explain the disparity between the Pax6 expression in different explants, with some explanted regions retaining Pax6 expression over time in culture, and others having lost Pax6 expression prior to dissection.

The expression of Pax6 in the untreated CS18 explant is not necessarily unexpected given that this phenomenon was relatively common in untreated embryonic chick explants cultured in the same manner, which did not undergo transdifferentiation. However, the fact that Pax6 expression was observed in some bFGF treated RPE cells of the CS18 explant (which exhibited evidence of transdifferentiation in other areas), which did not exhibit transdifferentiation in response to bFGF treatment, is more surprising given the apparent role of Pax6 maintenance in the maintenance in the capacity for transdifferentiation [as suggested by chick RPE studies (including chapter 3 & 4), as well as those in the newt (Kuriyama et al., 2009a, Spence et al., 2007b)]. This therefore implies that maintenance of Pax6 expression in RPE cells is not necessarily the critical indicator for the maintenance in the capacity for transdifferentiation. The likelihood given previous evidence would imply that Pax6 still has a crucial role to play in the induction of transdifferentiation, and in all likelihood, is still very important for the maintenance in the capacity for transdifferentiation in response to bFGF. However, other, currently unidentified regulatory elements must also be involved.

It is worthy of note that the expression of Pax6 in bFGF treated, CS18, RPE cells appears to be higher than that in untreated controls. This was observed in both transdifferentiated regions and RPE-like regions. This may suggest that bFGF treatment is able to up-regulate existing Pax6 expression as described for transdifferentiating embryonic chick and newt RPE cells (Spence et al., 2007b, Kuriyama et al., 2009a), without necessarily inducing transdifferentiation. Therefore, it may be that the action of Pax6 is attenuated in cells that expressed higher levels of Pax6, and retained their RPE phenotype. This is reported to be

the case in progressively maturing embryonic chicken RPE cells transfected with Pax6 *in vivo,* which can induce transdifferentiation (Azuma et al., 2005a) in an age-dependent manner. Similarly, the function of Pax6 is known to be very sensitive to differing levels of expression (Aota et al., 2003), with a number of both positive and negative feedback loops having been reported (Bharti et al., 2012). This may suggest that a threshold level of expression of Pax6 is required for the induction of transdifferentiation to occur. It is possible that regulatory pathways that attenuate bFGF signaling, the action of Pax6, or both, exist to prevent these cells from under-going transdifferentiation in response to bFGF treatment. Given the observed evidence, this seems highly likely. Therefore, elucidation of the identity of such pathways will be crucial to unlocking the potential of human RPE cells to undergo transdifferentiation. It is interesting to note that one of the CS19 explants which did not display evidence for transdifferentiation, did still weakly express Pax6 in the bFGF treated culture condition, which was absent from the negative control. This would suggest that bFGF was able to induce Pax6 expression in explanted CS19 RPE cells, but the potential effects of bFGF were not sufficient to induce transdifferentiation of the cells. This would perhaps support the idea of a threshold expression level of Pax6 being required for transdifferentiation to take place. Despite the clear difference of expression between treated and untreated RPE cells from this embryo, the increase in expression of Pax6 was absent from another CS19 explant. The reason for seems remains unclear, however, there may be a number of different explanations. As suspected for the Pax6 expression in CS18 explants, it may be possible that the observed Pax6 expression in bFGF treated cells of this CS19 explant did not result from the treatment with exogenous factors, but instead is an artifact of Pax6 expression in these particular RPE cells prior to dissection. No Pax6 expression in the untreated negative control culture may therefore result from a lack of Pax6 expression in a different area of the RPE sheet in this explant. If this is the case then the possible variability in the expression of Pax6 across different areas of the whole RPE monolayer would make it difficult to conclude the role of Pax6 in the capacity for transdifferentiation. However, it is also possible that the Pax6 observed in bFGF treated RPE cells may have also originally been present in untreated cells,

but has since become down-regulated over time in culture. If the same is happening in the bFGF treated cells, this is reflected in the weak labeling for Pax6. In this case, it is possible that the bFGF treatment is able to sustain a low level of expression of Pax6, possibly through counteraction of an unidentified mechanism responsible for intrinsic down-regulation of Pax6. It will be important to repeat the experiment should more human fetal tissue become available, in order to attempt to identify any trends in the expression of Pax6 which may indicate its role in transdifferentiation more clearly. Additionally, if possible, it would be useful to analyse the express patterns of Pax6 in both *en face* preparations of different stages of RPE, as well as the expression of Pax6 in post-dissected monolayers, in order to gain a clearer idea of the both the variability in the level of Pax6 expression in different regions of RPE with development, as well as any changes which may occur as a result of dissection.

5.3.4.2 CS19 RPE:

Despite not exhibiting transdifferentiation in this explanted RPE preparation, some evidence of CS19 RPE transdifferentiation was apparent in another bFGF treated explant. This included the characteristic presence of a non-pigmented neuroepithelium, continuous with pigmented RPE monolayer. This region was also observed to express neuro-retinal markers HuD and nestin. Unfortunately, no other sections were available in order to confirm the expression of the expected Pax6/Sox2 expression profile. Nonetheless, this is still compelling evidence of a level of transdifferentiation of the RPE having taken place. Interestingly, despite not exhibiting the same obvious morphological changes usually associated with transdifferentiation, this bFGF treated, CS19 explant did exhibit a very small amount of nestin expression in response to bFGF. This was also observed in CS21 explants in an earlier experiment (see experiment 5.2). This is particularly interesting because it suggests that despite the apparent lack of Pax6/Sox2 induction, some RPE cells were still able to respond to the exogenous bFGF signal by expressing a neural progenitor marker. This may imply that a portion of the bFGF signaling cascade required for neuronal differentiation is

active, but another arm of the cascade is being attenuated to prevent full transdifferentiation from taking place. If this is the case, it will be important to identify the inhibitory factors in order to unlock to potential of human RPE cells for transdifferentiation.

Despite the fact that Pax6 does not appear to be the ultimate marker of the capacity for transdifferentiation in response to bFGF, it still remains a useful marker of immature RPE cells which appear more likely to undergo the phenomenon, given its glaring presence in explants which have successfully undergone a limited amount of neural transdifferentiation.

It is unclear as to why there may be variation in the potential for transdifferentiation between two separate explants of the same stage, however, this is perhaps to be expected given comparisons with the chick model, where variation in the potential for bFGF-mediated transdifferentiation is also observed at later developmental stages comparable to CS19 (See chapter 3). It may also be possible that batch differences between the processing of each of the two explants could have occurred, which may have affected their relative development/reaction to the growth factor stimulus. For example, it is reasonable to assume that there may have been a degree of error in the original staging of each embryo prior to it being delivered, in addition, the untransdifferentiated explant may have taken longer to be delivered from the tissue bank, and therefore in real terms, be developmentally older than the explant which did exhibit some transdifferentiation. Similarly, one explant may have taken longer to prepare than another, which also introduces variability into the assay. However, at present, it remains unconfirmed as to why the process of transdifferentiation was observed to be variable in these explants.

5.3.4.3 CS22 RPE:

No transdifferentiation was observed in RPE explants taken from CS22, with barely detectable levels of nestin expression being present in response to bFGF, which may even be a background signal. No Pax6 expression was observed in treated or untreated RPE explants which suggests that bFGF is unable to induce, or maintain the expression of Pax6 in RPE

cells at this later stage. The expression of Pax6 in native RPE monolayers may be absent at this stage; therefore it would not be possible for it to be maintained. This does appear to be the case given the apparent absence of Pax6 expression in comparable stages of RPE *in vivo*. However, given the variability discussed earlier, this means that further replications of the experiment at this later developmental stage will be required in order to ascertain whether or not this result is consistent. If other CS22 explants do not exhibit transdifferentiation, the apparent trend where fewer explants are observed to undergo some transdifferentiation in response to bFGF with increasing developmental age, would tend to suggest that the capacity for transdifferentiation is developmentally restricted in human RPE cells, as in previously discussed avian/mammalian models.

5.3.4.4 Changes in pigmentation in response to bFGF:

Interestingly, despite the apparent developmental restriction in the capacity for transdifferentiation, some explants did appear to lose some of their pigmentation in response to bFGF, regardless of other evidence for transdifferentiation. Areas with a neuroepithelial-like morphology naturally contained the least amount of pigment, consistent with a retinal phenotype; however, they often retained a low number of small pigment granules, perhaps indicating their former specification as RPE cells. Other regions were more heavily pigmented, but bFGF treated explants did appear to be less so than untreated controls. In an effort to quantify this difference, the average pixel intensity value of each section was obtained from the confocal images. CS18 explants did show a statistically significant decrease in the level of expression of the RPE marker Pmel17, in addition to a statistically significant loss in pigmentation, both of which one would expect in RPE cells which are de-differentiating away from the RPE phenotype, perhaps in readiness to transdifferentiate towards a neuronal phenotype. Similarly, bFGF treated CS19 explants were observed to display a lighter phenotype than untreated controls, however this difference was not statistically significant. The expression levels of Pmel17 were not observed to be significantly

different. It might be expected that any pattern in the level of pigmentation would be reflected in the expression levels of Pmel17, however, as previously discussed, Pmel17 has not been found to reliably correlate with the level of pigmentation in RPE cells. The lack of a statistical significance in the difference in pigmentation between bFGF treated, and untreated explants may be an artifact of averaging of a low number of samples (n=2 where n = no. of biological repeats) with somewhat opposing phenotypes. The CS22 explant was surprisingly observed to display a statistically significant increase in Pmel17 expression in response to bFGF, in addition to a more expected decrease in the level of pigmentation. However, the region of interest sampling method used to obtain multiple values for a single explant (in order to try to account for the lack of biological repeats, n = 1) means that the standard error calculated for each culture condition is really more of a reflection of the variability in the expression of Pmel17, and the level of pigmentation, across a single section of explant. Therefore, this means that the statistically significant differences are actually mis-leading, and that further repetitions of the experiment are required in order to confidently ascertain whether CS22 RPE de-pigments in response to bFGF signaling in this culture system.

Nevertheless, it is interesting that some cells that do not exhibit transdifferentiation do appear to lose a degree of pigmentation at all stages investigated. Once again, this suggests that a degree of the bFGF signal is active in at least some cells, enough to initiate limited de-pigmentation. This would again support the idea that a different aspect of the bFGF signaling cascade is being attenuated in order to prevent transdifferentiation from taking place.

5.3.4.4 General discussion:

The transdifferentiation observed at the earlier stage primary human RPE was similar in appearance to that observed for later stage chicken RPE (approximately the equivalent stage to CS18) (See chapter 3). At these stages in chicken RPE, only particular regions were transdifferentiated and others retained the RPE phenotype. Therefore, this observation once again suggests that the relative capacity of RPE cells to transdifferentiate in response to bFGF

is conserved in development. This finding is important for future elucidation of the mechanisms that control the potential for transdifferentiation, as it would support the use of established animal models for the study of the phenomenon. Given the fact that transdifferentiation appears to be developmentally restricted to the earliest stages of development, it may be that HESC-RPE cells, which are known to maintain robust expression of Pax6 throughout their time *in vitro* (Vugler et al., 2008, Klimanskaya et al., 2004), and can be isolated almost as soon as they appear in culture, may retain the capacity for transdifferentiation. Therefore, given the likelihood that these human RPE cells are likely to be at an earlier developmental stage than other RPE cell lines, as well as the primary RPE tissue available, it is possible that these cells might display a greater propensity for transdifferentiation in response to bFGF treatment. The robust Pax6 expression maintained in HESC-RPE cells may imply that they are in fact developmentally arrested at an early developmental stage prior to the down-regulation of the transcription factor normally associated with the maturation of the RPE in several models of eye development, if not all (Spence et al., 2007b, Azuma et al., 2005a, Sakami et al., 2008, Arresta et al., 2005, Kuriyama et al., 2009b). Pax6 expression in HESC-RPE cells is only observed to be down-regulated following transplantation into the sub-retinal space, presumably where they continue their maturation (Vugler et al., 2008). This is of particular interest given the fact that Pax6 expression has been somewhat implicated with a retention in the capacity for transdifferentiation (Spence et al., 2007b, Kuriyama et al., 2009a, Idelson et al., 2009, Lu et al., 2009), if not the only factor involved as discussed previously. Maturation of the RPE may require additional signaling factors from surrounding tissues *in vivo*, for example, activin released from the adjacent ocular-mesenchyme (Fuhrmann et al., 2000b), which could possibly be the source of Pax6 inhibition.

The difference between the developing chick retina resulting from transdifferentiation, and the developing human retina resulting from transdifferentiation is marked, which is unsurprising given the gulf between their respective gestation periods (21 days for a chicken, 9 months for a human). After only 7 days in culture the transdifferentiated RPE has already

begun to display pseudo-stratification associated with the mature retina, in addition to expression of mature, cell specific markers such as rhodopsin, whereas human transdifferentiation only yields a small, apparently immature, retinal neuroepithelium, with no clear divide between developing retinal layers after 10 days. Despite this, the resulting human neuroepithelium does display the expression of ganglion cell marker HuD, after a 10 day culture period, which may suggest, as with rhodopsin expression in transdifferentiated chick RPE, that the human neuroepithelium can demonstrate accelerated development and expression of retinal cell specific markers, in comparison to what would be expected given the *in vivo* comparison. If this is in fact the case, then it is unclear why this would be, however, it may be related to the fact that transdifferentiated retinal tissue develops outside of the normal ocular environment, and therefore this system may lack the normal, regulatory signaling mechanisms that limit growth and development of the neural retina. It remains to be seen as to whether transdifferentiated retina can develop normally in the absence of these mechanisms, however, given that human RPE transdifferentiation appears to strongly reflect the development of transdifferentiated chick RPE, albeit on a different time-scale, the likelihood is that human RPE may also be capable of forming a more developed, retinal structure given time. This is even more likely to be the case should the developmental loss of the potential for RPE transdifferentiation be reversed, given that the retinal development of less mature RPE cells following transdifferentiation most closely aligns with that observed *in vivo*, with potentially a slight acceleration with respect to normal developmental time-scales.

Chapter 6 –

The potential for transdifferentiation in HESC-RPE cells

6.0 Introduction:

Human RPE cells appear to behave in a similar manner to that of embryonic chicken and rat cells, in that they appear to undergo transdifferentiation in response to bFGF at early stages of development. There is a problem in obtaining human RPE tissue at all, let alone tissue at the appropriate stages at which transdifferentiation is observed to occur. This is where the relatively recently discovered HESC-RPE could be very useful.

HESC-RPE could provide a sustainable source of human RPE cells for transdifferentiation. Furthermore, it should be possible to isolate HESC-RPE at the earliest stages of development when transdifferentiation is more likely to take place, owing to the fact that it can be readily identified via its pigmented phenotype. HESC-RPE is also known to robustly express Pax6 when expanded *in vitro* (Vugler et al., 2008, Lund et al., 2006b, Idelson et al., 2009, Liao et al., 2010, Lu et al., 2009, Klimanskaya et al., 2004) and this expression is only lost following transplantation of the HESC-RPE cells into the sub-retinal space (Vugler et al., 2008). This would suggest that HESC-RPE cells *in vitro* are developmentally arrested, and are suspended in an immature state, given the fact that down-regulation of Pax6 in the RPE is associated with the on-going maturation of the RPE in several species, if not all species (Spence et al., 2007b). Maturation of the RPE may require additional signaling factors from surrounding tissues *in vivo*, for example, activin released from the adjacent ocular-mesenchyme (Fuhrmann et al., 2000b), which could possibly be the source of Pax6 inhibition. If HESC-RPE cells are developmentally arrested, this would be very encouraging for their potential to undergo transdifferentiation in response to bFGF. Importantly, HESC-RPE would then provide a fantastic source of human retinal tissue because it can be readily expanded *in vitro* without losing the expression on Pax6, and therefore it isn't difficult to obtain a large amount of source material from which transdifferentiated retina could be obtained. This is in contrast to recent reports which produce human retinal tissue through the step-wise differentiation of undifferentiated human stem cells (embryonic and iPS). These protocols require aggregates of a certain size in order to produce retinal tissue (Eiraku and Sasai, 2012b,

Meyer et al., 2011, Osakada et al., 2009), which may require multiple, complicated, individual experiments in order to produce enough useful material, whereas transdifferentiation of the RPE could potentially be much simpler.

The earliest stage at which intact HESC-RPE sheets could be isolated was immediately following genesis of the pigmented foci in super-confluent flasks of HESCs. Given the likelihood that the most immature RPE cells are those which are most likely to undergo transdifferentiation in response to bFGF, these intact foci were cultured with bFGF as soon as they could be isolated.

Additionally, cultures of HESC-RPE monolayers expanded over time have been reported to grow in the presence of large, colourless, lens-like structures, referred to as lentoids, in addition to expressing αA-crystallin. This is encouraging for their potential to undergo transdifferentiation.

6.1 Characterisation of HESC-RPE cells in culture:

6.1.2 Introduction:

Given the fact that RPE cells have been reported to undergo transdifferentiation towards a lens phenotype, in addition to a neural retinal phenotype, the expression of αA-crystallin, the major structural protein competent found in the lens (Fujii et al., 2003), was investigated in cultured HESC-RPE monolayers. It was also necessary to investigate the expression of the FGF-R1 in HESC-RPE monolayers given the fact that the ability to detect bFGF is a crucial induction factor in the initiation of transdifferentiation, and a loss in expression has been suggested as a reason for the loss in capacity for transdifferentiation (Spence et al., 2004).

6.1.2 Materials & Methods:

6.1.2.1 Generation & isolation of human embryonic stem cell-derived RPE:

HESC-RPE was derived as described in chapter 2.10.

6.1.2.2 Culture of HESC-RPE cells:

HESC-RPE foci were encouraged to form monolayers by culture on Growth Factor-reduced Matrigel Basement Membrane Matrix (BD Biosciences. Matrigel was prepared by diluting 1:30 in ice cold KnockOut^{TM} DMEM inside a chilled centrifugation tube. The diluted solution was applied to tissue culture plates using chilled stripettes. Plates were incubated overnight at 4^{o}C or for at least 30mins at 37^{o}C before use. Coated plates were also stored for up to 10 days at 4°C according to the manufacturer's instructions. Any unbound Matrigel was aspirated from the tissue culture plastic immediately before use. HESC-RPE foci were then seeded with care taken to place HESC-RPE sheets basal side down where possible. HESC-RPE cell were expanded in HESC-medium –bFGF for 5 weeks at 37°C + 5% CO_2 with spent media replaced approximately twice a week.

6.1.2.3 *Immunohistochemistry and statistical analysis:*

Immunohistochemistry (chapter 2.4), image analysis (chapter 2.5/5.3.2.3) and statistical analysis (chapter 2.11) was performed as previously described.

6.1.3 Results:

Pmel17 positive, pigmented HESC-RPE monolayers were observed to express αA-crystallin throughout the cytoplasm of many of the cells in the monolayer (Fig. 6.1). All the cells in the monolayer were observed to express RPE marker Pmel17 (Fig. 6.1A), in addition to varying levels of pigmentation (Fig. 6.1B). All cells in the monolayer also appeared to display the characteristic, epithelial, polygonal morphology normally associated with RPE cells (Fig. 6.1B,E), which removes the possibility of contaminant cells being present. αA-crystallin expression was observed at high magnification to confirm co-localisation of the protein with Pmel17 expression (Fig. 6.1C, D yellow arrows), as well as polygonal, pigmented RPE cells (Fig. 6.1E).

It has been suggested that RPE cells may eventually down-regulate the expression of bFGF receptor, fibroblast growth factor-1 (FGF-R1), at later stages of development. This could possibly be a reason for the apparent loss of response to bFGF treatment at later developmental stages, and consequently a loss in the potential for transdifferentiation. It was therefore necessary to investigate the expression of FGF-R1 in HESC-RPE cells in order to ascertain whether or not it was expressed. FGF-R1 was robustly expressed in all HESC-RPE cells, as analysed using immunohistochemistry (Fig. 6.2A). The antibody labelled the cell membrane, which is where the FGF-R1 protein is localised (Fig. 6.2A). The antibody could only label the outside of cells given the fact that no Triton-X detergent was used to permeablize the cells during antibody labelling. In order to confirm the specificity of the antibody labelling, RT-PCR was employed to confirm the expression of FGF-R1 in HESC-RPE cells. As expected, FGF-R1 was robustly expressed in HESC-RPE cells as demonstrated by a bright band of the correct size (614bp) for the expected PCR product (Fig. 6.2C). The identity of the PCR product was subsequently confirmed to be FGF-R1 by sequencing.

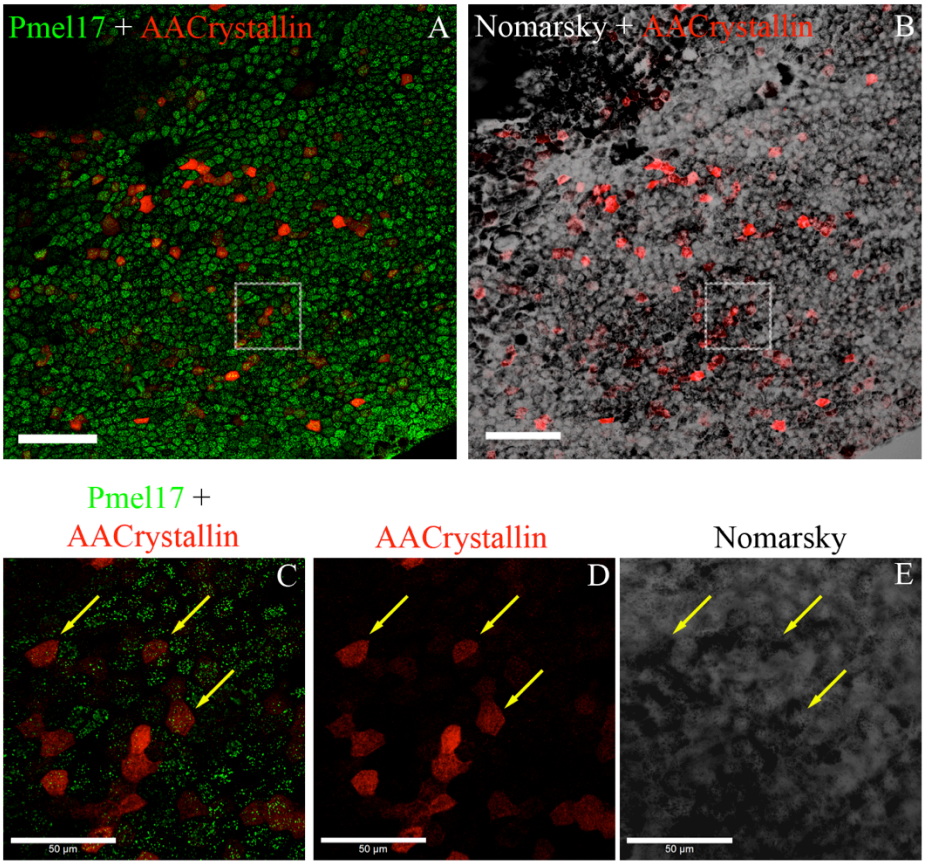


Fig. 6.1
Expression of lens marker in a HESC-RPE monolayer on Matrigel.
(A, B) Pmel17 (green) positive, pigmented HESC-RPE cells which display a characteristically polygonl morphology are observed to express alphaA-Crystallin (red), a lens-associated/chaperone protein, in a number of cells in the monolayer. alphaA-crystallin expression was observed to co-localise with both Pmel17 (A, C yellow arrows), and pigmentation (E yellow arrows). C-E is high mag. white boxes from A, B. Scale bars: (A, B) 100uM, (C-E) 50uM.

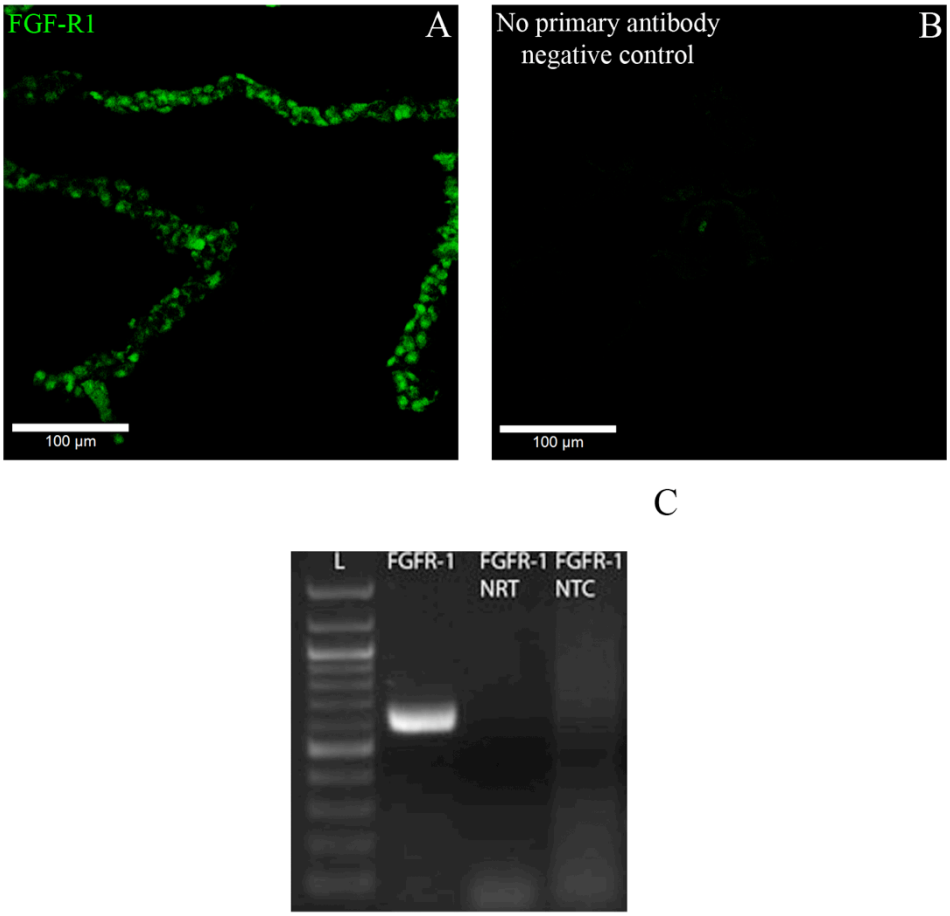


Fig. 6.2
Expression of FGFR-1 in HESC-RPE monolayers explanded on Matrigel. FGFR-1 (green) was robustly expressed in all HESC-RPE cells in section (A). This was confirmed by PCR which revealed a clear band at 614bp (C) with no equivalent products in the no reverse transcriptase (NRT), or NTC (no template control) negative controls. Only background signal was detected in the no primary antibody negative control (B). This suggests that HESC-RPE may still be able to respond to exogenous bFGF. Scale bars: 100uM.

No equivalent bands were present in either of the negative controls which confirms that the positive band in the reverse transcriptase reaction has resulted from cloning of FGF-R1 cDNA produced from FGF-R1 transcripts in the HESC-RPE, and not from genomic or other DNA contamination (Fig. 6.2C FGFR-1 NRT). Similarly, absence of a band in the no template control (Fig. 6.2C NTC) confirms that the product in the reverse transcriptase reaction were not a result of cloning of DNA contaminants in the PCR reagents.

6.1.4 Discussion:

If the capacity for transdifferentiation is developmentally restricted in human RPE cells, as appears to be the case, HESC-RPE cells may provide the best source of cells to study the phenomenon in human RPE owing to the fact that they are likely to be the most developmentally immature, sustainable source of human RPE cells available. Especially given the difficulties in obtaining human RPE cells at all. Additionally, there have been some reports that cultures of HESC-RPE cells have been observed to contain colourless, lens-like structures known as 'lentoids,' which may have resulted from the transdifferentiation of the HESC-RPE cells towards a lens phenotype, rather than a neuronal phenotype. A lens phenotype is the alternative differentiation lineage transdifferentiating RPE cells are able to follow (Kodama and Eguchi, 1995, Ooto et al., 2003, Hyuga et al., 1993). Despite the lack of visible lentoid structures, many of the HESC-RPE cells in a cultured monolayer were observed to express αA-crystallin, the major structural component of the lens (van Boekel et al., 1999, Fujii et al., 2003), throughout the cytoplasm. This expression was co-localised with RPE marker Pmel17, as well as intense pigmentation characteristic of RPE cells. This is surprising because αA-crystallin is not usually expressed outside of the lens itself, and particularly not inside RPE cells. This finding may be encouraging for the potential plasticity of HESC-RPE cells given that they appear to be expressing a marker associated with transdifferentiation. However, crystallins are also reported to act as molecular chaperones,

which are able to provide a number of functions, including acting as heat shock proteins (van den et al., 1996, Andley et al., 1996, Sun et al., 1997, Cherian-Shaw et al., 1999, Das et al., 1999, van Boekel et al., 1999, Fujii et al., 2003, Kundu et al., 2007). Therefore, the presence of αA-crystallin in HESC-RPE may also result from an unidentified need for molecular chaperones, possibly as a result of cellular stress brought about by a culture medium that is optimized for HESC's, not RPE cells. Additionally, αA-crystallin expression may also be an artifact of the sustained expression of Pax6 in HESC-RPE monolayers, given that Pax6 is known to be a crucial regulator of αA-crystallin expression(Cvekl et al., 1995, Cvekl et al., 1994, Cvekl et al., 2004, Yang et al., 2006).

It has been suggested that a down-regulation in the expression of the bFGF receptor, FGF-R1 may be responsible for the loss in the potential for transdifferentiation of RPE cells in response to bFGF (Spence et al., 2004). However, this now seems unlikely given the fact that the expression of some neuronal markers, in addition to de-pigmentation of RPE explants, have been observed in response to bFGF (Chapter 5). Nevertheless, it was important to test this hypothesis in case it was found to be responsible for a loss in the capacity for transdifferentiation. As expected, HESC-RPE cells were observed to robustly express FGF-R1 in all cell membranes, which is characteristic of a membrane-localised receptor protein. Therefore, it is reasonable to assume that a loss in the capacity for transdifferentiation was not as a result in the loss of the bFGF receptor.

6.2 The potential for transdifferentiation of HESC-RPE cells:

6.2.1 Introduction:

Given the fact that HESC-RPE may be developmentally arrested, and that in theory, these are the earliest available, sustainable source of RPE cells which can be obtained, it could be argued that they are likely to exhibit to greatest potential for transdifferentiation of

all available sources of human RPE. Their potential to undergo transdifferentiation in response to bFGF was therefore tested. Additionally, given the fact that inhibition of the activin signalling pathway has been shown to increase the capacity for transdifferentiation in response to bFGF at later stages of development in embryonic chick RPE cultures (Sakami et al., 2008), both through extension of the window of responsiveness to bFGF, and also through a reversal of the loss in sensitivity to bFGF after the RPE has apparently lost the potential for transdifferentiation. This effect has been observed to increase the potential for transdifferentiation in RPE cells up to 2 days older than 'bFGF only' treated cells. If a similar effect can be replicated in human RPE cells, even a limited developmental extension in the capacity for transdifferentiation, comparable to that reported in the chick, would allow human RPE cells to undergo transdifferentiation at approximately 1 week older in development than expected. This extension of the transdifferentiation window may be sufficiently long to allow most HESC-RPE cells to undergo the phenomenon following isolation after around 4/5 weeks *in vitro* development. It was therefore necessary to observe whether or not a similar effect could be replicated in HESC-RPE cultures.

6.2.2 Materials & Methods:

6.2.2.1 Production and culture of HESC-RPE:

HESC-RPE foci were produced as described in chapter 2. Non-adherent cultures of intact HESC-RPE foci from the earliest stage possible following genesis (approximately within 1 week) were mechanically isolated and cultured in the standard, non-adherent, transdifferentiation culture system +/- 100ng/ml bFGF in the same manner previously utilized for primary human fetal RPE transdifferentiation studies (Chapter 5).

Activin signalling inhibitor, SB431542, was employed in order to attempt to increase the capacity from transdifferentiation of HESC-RPE in culture. bFGF treated cultures were also treated with 14μM SB431542, a concentration which had previously been reported to

extend the capacity for transdifferentiation (Sakami et al., 2008). These cultures were supplemented with fresh inhibitor every day until the end of the culture, whereas negative controls were treated with the equivalent volume of DMSO.

6.2.2.2 Immunohistochemistry and statistics:

Immunohistochemistry statistical protocols are described in chapter 2.4/2.11 respectively.

6.2.2.3 Image analysis:

Image analysis protocol was the same used for multiple sections of biological repeats as described in chapter 5 (see Fig. 5.M3).

6.2.3 Results:

6.2.3.1 Treatment with bFGF only:

The majority of HESC-RPE foci treated with bFGF in the standard, non-adherent culture system, did not exhibit any evidence that transdifferentiation towards a neural retinal phenotype (n=6)(Fig. 6.3). Both bFGF treated and untreated HESC-RPE foci retained their characteristic pigmentation and monolayer phenotype (Fig. 6.3A, F, K, O) as observed via nomarsky optics and DAPI nuclear staining, respectively. In keeping with this retention of the RPE phenotype, bFGF treated foci expressed RPE marker Pmel17 at an apparently equivalent level to that of untreated, negative controls (Fig. 6.3E, J). The majority of cultured HESC-RPE cells displayed a characteristically RPE-like phenotype and did not express markers usually associated with transdifferentiated neural retina as expected (Fig. 6.3). This was consistent for both bFGF treated, or untreated HESC-RPE cultures (Fig. 6.3), and included the absence of neural progenitor marker, Sox2 (Fig. 6.3C, H), ganglion/amacrine cell marker

HuD (Fig. 6.3N, R) and photoreceptor marker, rhodopsin (Fig. 6.3M, Q). Additionally, Pax6, a transcription factor heavily implicated in the induction of transdifferentiation, was absent from HESC-RPE in both culture conditions (Fig. 6.3D, I).

However, one of the HESC-RPE foci treated with bFGF did exhibit some evidence of transdifferentiation of the RPE towards a developing neuroepithelial phenotype (Fig. 6.4). The evidence for transdifferentiation was similar in appearance to that observed in bFGF treated, RPE cultures at later developmental stages using chick RPE explants, as well as the earliest stages of human fetal RPE explants available. This HESC-RPE aggregate treated with bFGF was observed to have a region of the RPE monolayer on the surface of the aggregate, which was thicker in appearance than the rest of the RPE monolayer, and appeared to consist of multiple layer of cells, given the overlapping nuclei resent in this region (Fig. 6.4A-E yellow arrows, F-L). This HESC-RPE aggregate was observed to express a number of markers usually associated with retinal tissue resulting from transdifferentiation.

A large proportion of cells attached to the basal side of the HESC-RPE monolayer were positive for the expression of Sox2 (Fig. 6.4D), which suggests that these cells are likely to be neural progenitors. Many of these Sox2 positive cells at the basal surface of the HESC-RPE were also positive for Pax6 expression (Fig. 6.4C). The Sox2 positive cells found immediately adjacent to the region of thickened HESC-RPE (Fig. 6.4A-E yellow arrows) were not found to express Pax6, which was largely confined to a different area of the aggregate. The thickened region of HESC-RPE was also observed to express developing retinal markers usually associated with the early stages of transdifferentiation of RPE cells. The nuclei in this region were robustly labelled for Pax6 (Fig. 6.4B, C, F- I), in addition to neural progenitor marker Sox2 (Fig. 6.4B, D, F-H, J) in a multi-layered fashion characteristic of a presumptive retina in development. Both of these markers were absent from the rest of the HESC-RPE cells in the aggregate, which is consistent with retention of the RPE phenotype (Fig. 6.4A-E). Pax6 positive cells in the multi-layered region were also co-localised with Sox2 expression, in all cells of the thickened region (Fig 6.4H).

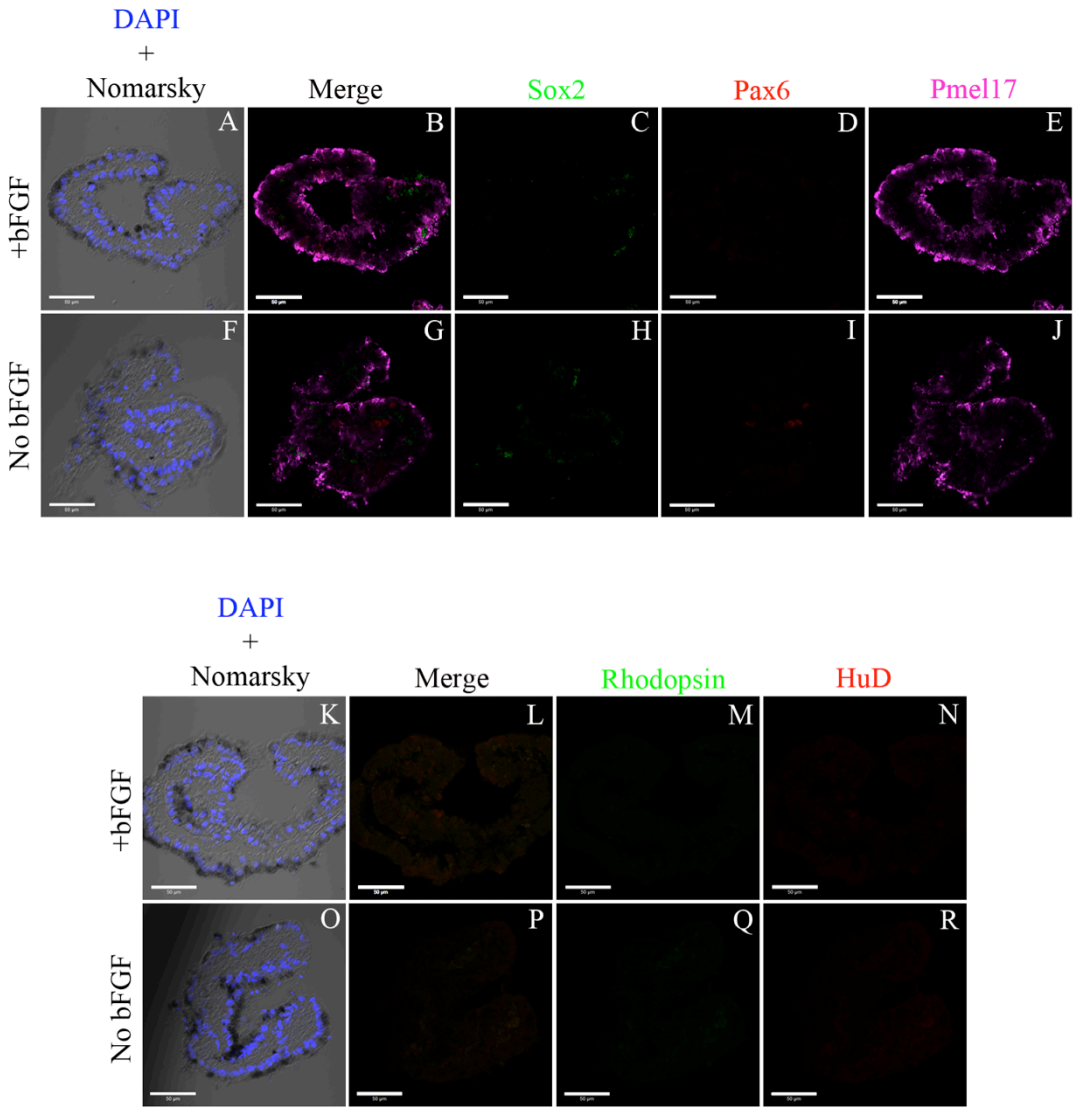


Fig. 6.3
HESC-RPE foci cultured as non-adherent aggregates treated +/-bFGF (100ng/ml) for 10 days - no transdifferentiation.
The majority of HESC-RPE foci treated with bFGF did not display evidence of transdifferentiation. (A, E, F, J, K, O) These foci retained a characteristically Pmel17 (purple) positive, pigmented, monolayer phenotype. No retinal markers including: Sox2 (C, H) (green), Pax6 (D, I) (red), HuD (N, R) (red), or rhodopsin (M, Q) (green) were expressed in bFGF treated, or untreated foci. Scale bars: 50uM.

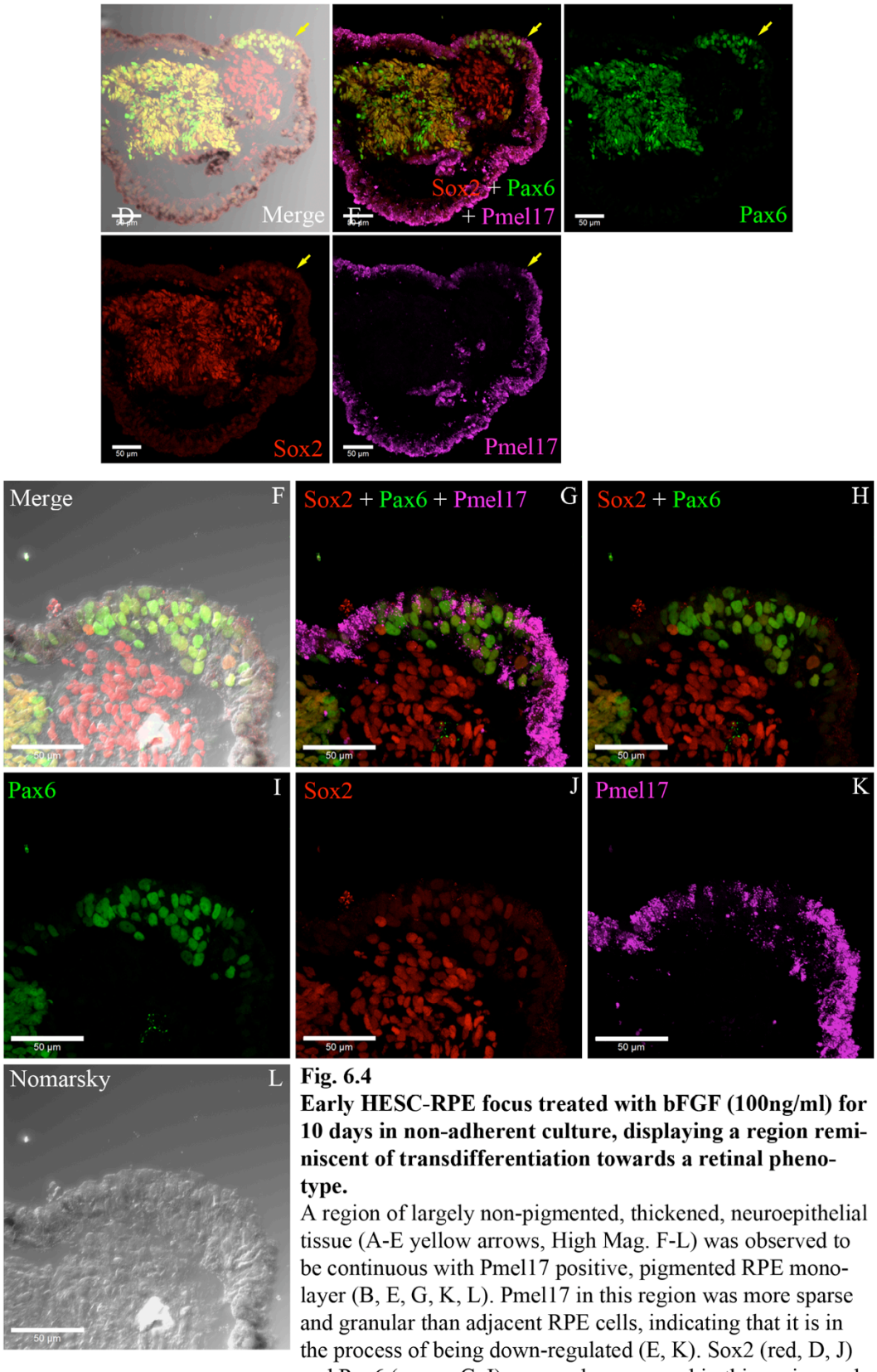


Fig. 6.4
Early HESC-RPE focus treated with bFGF (100ng/ml) for 10 days in non-adherent culture, displaying a region reminiscent of transdifferentiation towards a retinal phenotype.
A region of largely non-pigmented, thickened, neuroepithelial tissue (A-E yellow arrows, High Mag. F-L) was observed to be continuous with Pmel17 positive, pigmented RPE monolayer (B, E, G, K, L). Pmel17 in this region was more sparse and granular than adjacent RPE cells, indicating that it is in the process of being down-regulated (E, K). Sox2 (red, D, J) and Pax6 (green, C, I) were only expressed in this region and were observed to co-localise as in retinal progenitors (A, B, F, H). Scale bars: 50uM.

Consistent with the idea that most of the aggregate has retained the standard HESC-RPE phenotype, these cells, which exist as a monolayer, were observed to retain their heavy pigmentation (Fig. 6.4A) as well as robust expression of RPE marker Pmel17 (Fig. 6.4E). In contrast, the thickened region of cells which express markers of early retinal development, did not appear to be pigmented like the adjacent HESC-RPE cells, but appeared to have lost most of their pigmentation, with only a few very small pigment granules remaining (Fig 4L). Pmel17 expression appeared to have been down-regulated in this region of cells (Fig. 6.4K), which is consistent with this apparent loss of the RPE phenotype in this region, and a move towards a neuroretinal phenotype. Pmel17 expression appeared highest at the points where the retinal region was continuous with the adjacent, heavily pigmented, HESC-RPE monolayer (Fig. 6.4E, K). Pmel17 expression was then reduced in somewhat of a gradient towards the thickest point of the retinal region, where some cells were negative for the expression of the RPE marker (Fig. 6.4K). At this point, the Pmel17 expression which was present appeared to be very granular and sparse (Fig. 6.4K) than in adjacent RPE cell, in a similar manner to Pmel17 expression observed in the human fetal retina, both in central, and peripheral (CMZ) regions at the border of pigmented RPE and retinal tissue (Chapter 5, Figs. 5.2-5.5). Crucially, the fact that this apparently neural retinal region of cells was continuous with the pigmented, Pmel17 positive, HESC-RPE monolayer, suggests that it may have resulted from transdifferentiating HESC-RPE cells. Unfortunately, no additional tissue sections were available for further analysis of this apparently transdifferentiated retinal region.

However, another untreated HESC-RPE aggregate also displayed evidence of RPE to neural retina transdifferentiation, in a similar proportion (1 aggregate of n=5) tested in the transdifferentiation culture system (Fig. 6.5). This HESC-RPE culture displayed a large region of multi-layered, neuroepithelial like cells, as viewed using a DAPI nuclear stain (Fig. 6.5A, F), which was continuous with a single layer of heavily pigmented (Fig. 6.5A, F), Pmel17 positive (Fig. 6.5B, E) HESC-RPE cells at both ends. No other contaminant tissue appeared to be present at the basal surface of the HESC-RPE aggregate (Fig. 6.5A-J). Interestingly, this retinal region was also observed to lack pigmentation when compared to the

adjacent HESC-RPE monolayer region, with only very few pigment granule remaining in the neuroepithelium (Fig. 6.5A, F), and consistent with this observation, these cells were also negative for Pmel17 expression (Fig. 6.5B, E). Pmel17 ceased to be expressed at the border between the pigmented HESC-RPE monolayer and the non-pigmented neuroepithelium (Fig. 6.5B, E yellow arrows). The nuclei in this neuroepithelium were observed to express markers of developing retinal progenitors: Pax6 (Fig. 6.5D) and Sox2 (Fig. 6.5C), which were absent from Pmel17 positive, pigmented HESC-RPE cells. The only signal detectable in pigmented regions was autoflurorescent background material, which was not localised to the nucleus of the cell where Pax6 and Sox2 transcription factors are located. The majority of Pax6 and Sox2 positive cells in the retinal neuroepithelium were observe to express both of the transcription factors, as observed via co-localisation of the antibody labelling for both markers (Fig. 6.5B).

An adjacent section analysed for the expression of specific retinal cell markers supported the idea that RPE had undergone transdifferentiation towards a neuro-retinal phenotype (Fig. 6.5F-J). Amacrine and ganglion cell marker, HuD, expression was observed in the neuroepithelial region which was first observed at the junction between pigmented HESC-RPE cells and the non-pigmented neuroepithelium (Fig. 6.5F-H, J yellow arrows). HuD expression was absent from pigmented HESC-RPE cells as expected for cells that retain the characteristic epithelial RPE phenotype (Fig. 6.5F-H, J). The most robust expression of HuD was observed in the cytoplasm of cells at the basal side of the neuroepithelium where one would expect to find the ganglion cells in an inverted transdifferentiated retina (Fig. 6.5G-H). A small number of other cells also expressed HuD at a lower level and were located in the middle of the neuroepithelial region (Fig. 6.5G, H). These cells appeared to have a long, neuronal morphology, with a perpendicular orientation, which is characteristic of neuronal cells in the retina (Fig. 6.5G, H). It is likely that these HuD positive cells located in the middle of the neuroepithelium were ganglion cells migrating to the surface of the developing retina, and/or amacrine cells. No rhodopsin cells were present in any region of the HESC-RPE aggregate, neuroepithelial or RPE-like in phenotype (Fig 6.5G, I).

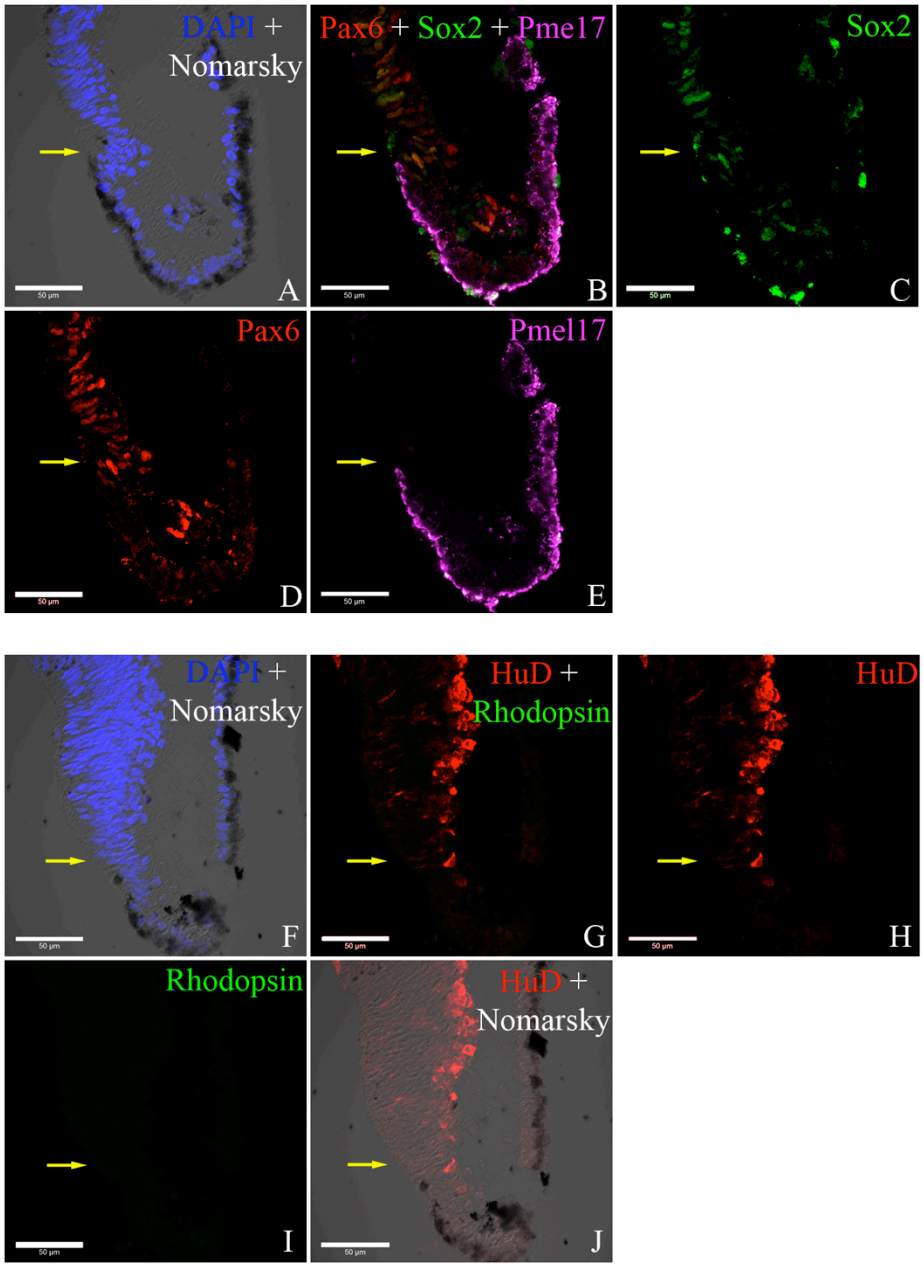
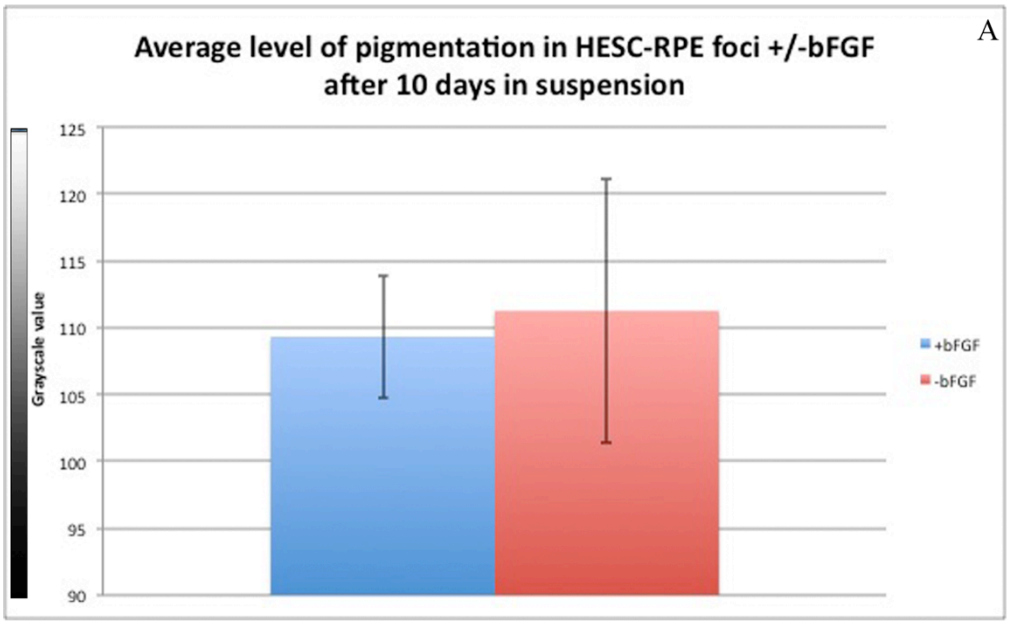


Fig. 6.5
Evidence for transdifferentiation of HESC-RPE focus cultured aggregate in non-adherent conditions for 10 days without treatment with bFGF.
Despite having not been treated with exogenous growth factors, one HESC-RPE aggregate still displayed evidence for transdifferentiation of the RPE having taken place. A non-pigmented, thickened, neuroepithelial structure was observed to be continuous (A, F yellow arrows) with a pigmented, RPE monolayer (A, F, E). This neuroepithelial structure was observed to express both Pax6 (D) and Sox2 (C), which co-localised in many cells (B), implying a retinal progenitor phenotype. Additionally, it also expressed HuD (G, J) which localised to the basal side of the presumtpive retina in the developing ganglion cells, indicating an inverted phenotype. This is characteristic of transdifferentiated retina. No rhodopsin expression was observed (I). Pigmented RPE did not express any retinal markers (A-J). Scale bars: 50uM.



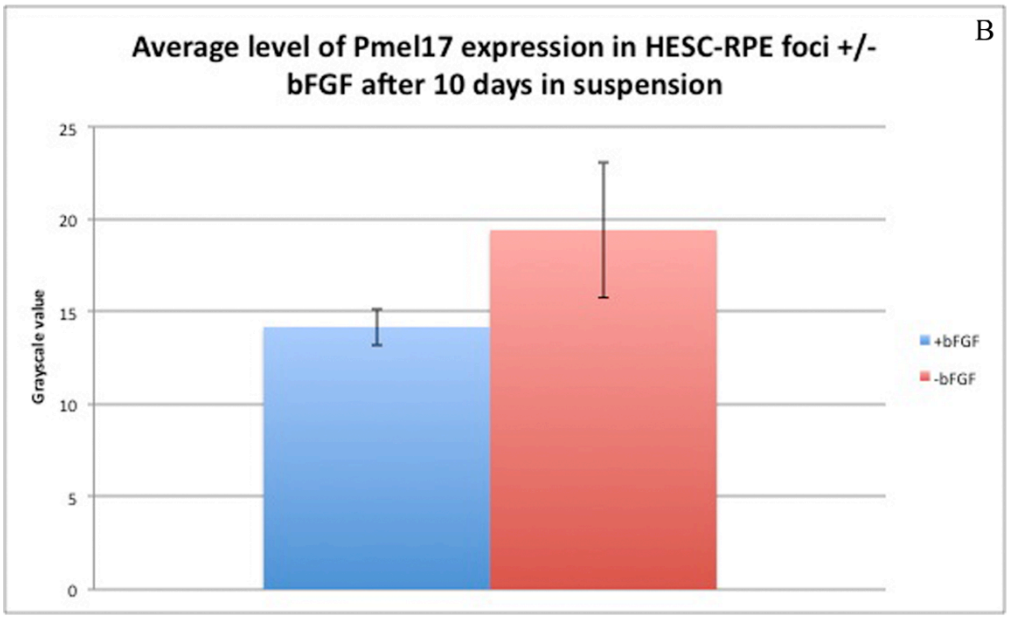


Fig. 6.6
Quantification of the level of expression of Pmel17, and the level of pigmentation in HESC-RPE foci treated +/-bFGF (100ng/ml) after 10 days in non-adherent culture, using image analysis software.
The average expression/pigmentation level was calculated by analysing the average pixel intensity of grayscale images for multiple images in each culture condition. The differences in expression/pigmentation level are therefore expressed on a grayscale (Black = 0, White = 255). (A) No significant difference in the level of pigmentation was observed between bFGF treated (Blue bars), and untreated (Red bars) HESC-RPE cultures. (B) The level of expression of Pmel17 appears to be significantly less in bFGF treated HESC-RPE compared with untreated controls, however, this difference was not found to be statistically significant (RANOVA). Error bars: Standard error.

Quantification of the level of pigmentation and level of expression of Pmel17 in bFGF treated (n=6) and untreated (n=5) HESC-RPE cultures using image analysis software was employed to see if bFGF treatment was able to effect these variables, which will hopefully highlight whether or not the growth factor is able to effect the normal RPE phenotype. The level of pigmentation was found to be relatively similar in both bFGF treated, and untreated HESC-RPE cultures, with no significant difference being observed between the two culture conditions (Fig. 6.6A). Interestingly, bFGF treatment was observed to reduce the expression level of RPE marker, Pmel17, compared with untreated negative controls (Fig. 6.6B), however, his difference was not observed to be statistically significant (RANOVA).

6.2.3.2 Treatment with bFGF + activin inhibitor (SB431542):

Blockage of the activin signalling pathway using a pharmacological inhibitor, SB431542, in addition to bFGF treatment, did not result in a greater proportion of HESC-RPE aggregates exhibiting evidence of transdifferentiation (n=5) than DMSO treated negative controls (n=5) (Fig. 6.7). The majority of these cultures were observed to maintain their characteristic, pigmented RPE, monolayer phenotype regardless of the treatment with SB431542+bFGF or DMSO only (Fig. 6.7A, F, K, O). In keeping with this, both SB431542_bFGF and DMSO controls were both observed to express Pmel17, as one would expect from cells that retain the RPE phenotype. All HESC-RPE cultures were negative for developing retinal markers, Sox2 (Fig. 6.7C, H), and Pax6 (Fig. 6.7D, I), as well as markers of specific retinal cell populations, HuD (Fig. 6.7N, R) and rhodopsin (Fig. 6.7M, Q). However, as with the previous experiment, which employed bFGF treatment only, one HESC-RPE aggregate treated with SB431542+bFGF did display some characteristics of transdifferentiation towards a neural retina phenotype (Fig. 6.8). This HESC-RPE aggregate displayed a region with decreased levels of pigmentation in comparison to adjacent RPE cells, with which it was continuous (Fig. 6.8A, F, J). This region still displayed a number of patches

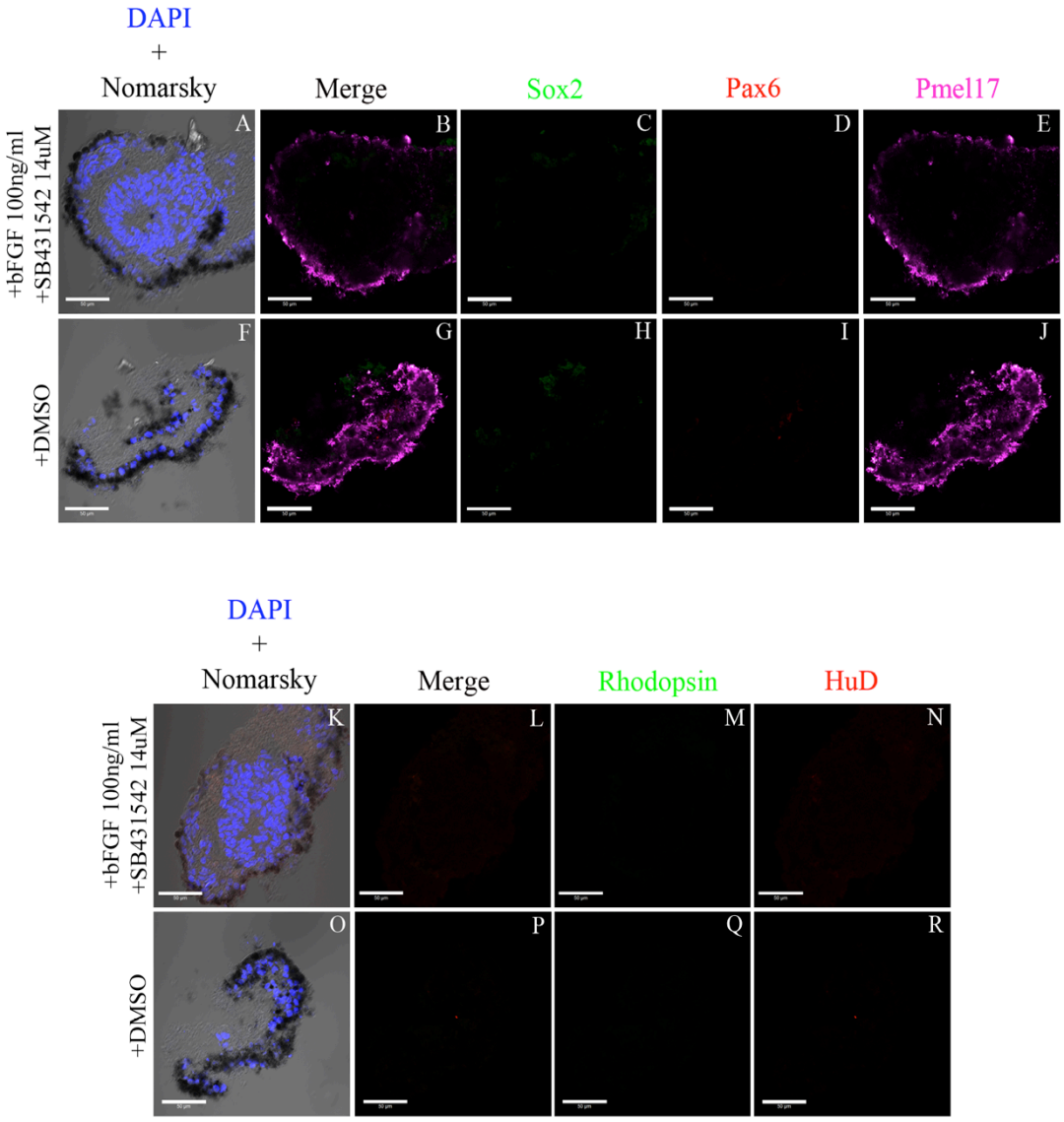


Fig. 6.7
HESC-RPE foci treated with bFGF (100ng/ml) + Activin inhibitor SB431542 (14uM), or DMSO negative control, in non-adherent culture conditions for 10 days - no transdifferentiation.
The majority of aggregates treated with bFGF+SB431542 (activin signaling inhibitor) did not display evidence of transdifferentiation. These aggregates retained a pigmented RPE phenotype (A, F, K, O) and expressed RPE marker, Pmel17 (E, J). No retinal markers, including: Sox2 (C, H), Pax6 (D, I), HuD (N, R) or rhodopsin (M, Q) were observed in these cultures. Together, this would support the idea that HESC-RPE have retained their phenotype following the culture period, regardless of treatment with bFGF. Scale bars: 50uM.

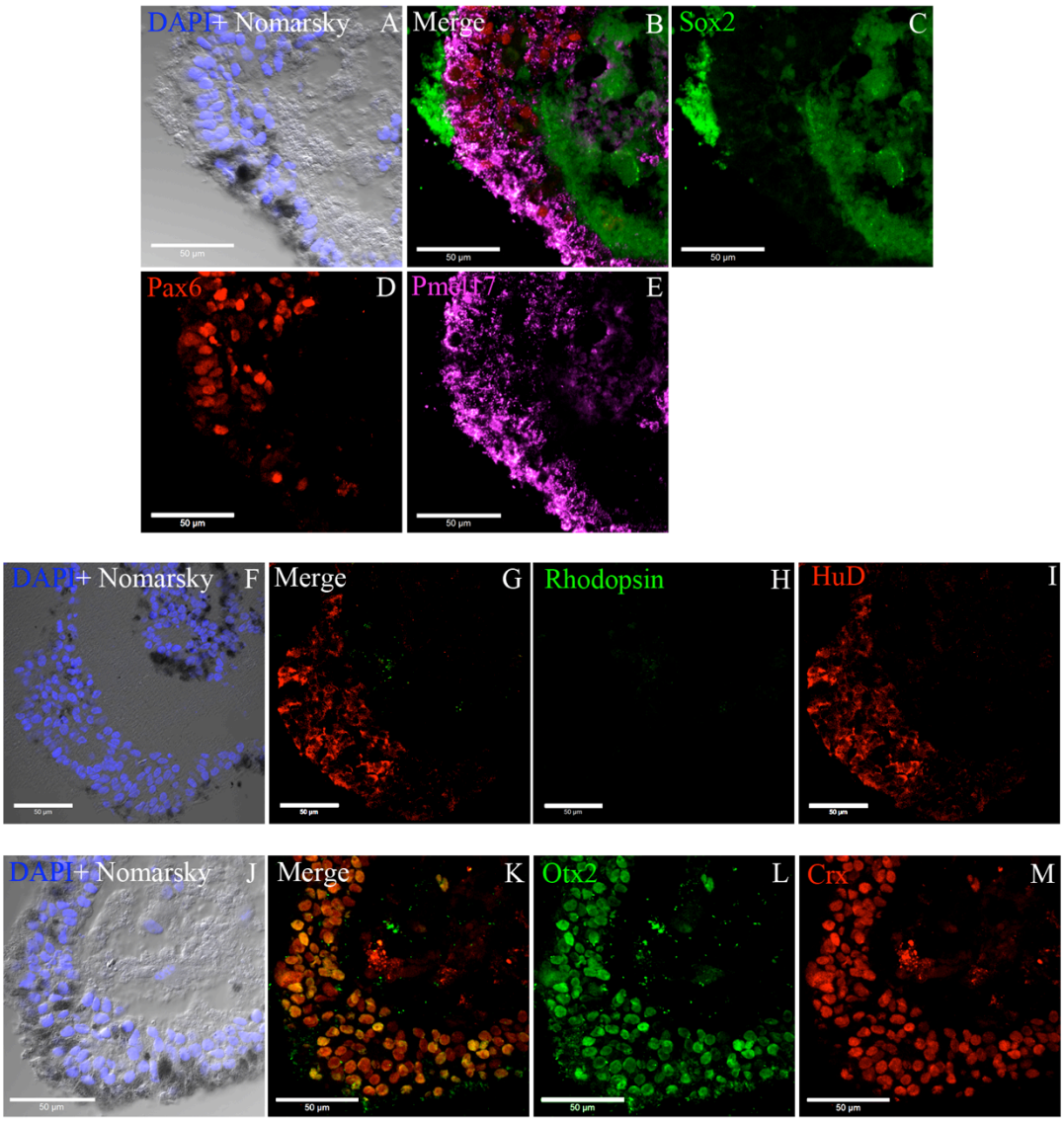


Fig. 6.8
HESC-RPE aggregate treated with bFGF (100ng/ml) + Activin signaling inhibitor SB431542 (14uM), or DMSO, after 10 days in non-adherent culture.
This bFGF + activin signaling inhibitor treated aggregate did appear to exhibit evidence of a limited amount of transdifferentiation. A region of neuroepithelial-like, thickened tissue was observed to be continuous with the pigmented RPE monolayer (A, F, J). This region retained the expression of RPE marker Pmel17 (B, E), however, this appeared to be more granular in some of the thickened region compared with the adjacent RPE monolayer. Many cells in this region were observed to express Pax6 (D), and Sox2 (C), which co-localised on some occasions, indicating the presence of retinal progenitor cells in this region. No Sox2 or Pax6 were expressed outside of this reigon. In addition to retinal progenitor markers, some weak HuD expression was observed throughout the retina-like region (G, I), however, no rhodopsin expression was observed (H). This would suggest that a degree of transdifferentiation of HESC-RPE towards a neuronal phenotype has occurred. The region was also labelled for both Otx2 (L) and CRX (M) in all cells of this region (K). Fluorescence digitally enhanced. Scale bars: 50uM

of pigmentation despite the apparent loss of pigmentation. As one would expect from a transdifferentiated region of RPE, cells in this region appeared to be multi-layered as observed via DAPI, nuclear staining (Fig. 6.8A, F, J). Interestingly, this region also expressed similar levels of Pmel17 as adjacent regions with more pigmentation (Fig. 6.8B, E), however, despite this, a number of markers of retinal neuroepithelium were also expressed. Many of the nuclei in this region were labelled for Pax6 expression (Fig. 6.8D), and a number of other cells were also labelled for Sox2 expression (Fig. 6.8C). Some of these Pax6 positive cells were also positive for Sox2 expression (Fig. 6.8B), which suggests that they are retinal progenitor cells, although, not all of the Pax6 positive cells were positive for Pax6. Heavily pigmented cells outside the neuroepithelial region were negative for both Pax6 and Sox2 expression as expected for Pmel17 positive RPE cells (Fig. 6.8C, D). In addition to these retinal progenitor markers, this region also exhibited a large number of cells that expressed HuD (Fig. 6.8G, I). These cells were weakly labelled for HuD and they were located throughout the breadth of the apparently neuroepithelial layer, with no specific localisation which might support the hypothesis that this region resulted from transdifferentiated HESC-RPE (like the HuD in Fig. 6.5). No rhodopsin expression was observed in any region of the aggregate (Fig. 6.8H). Fortunately, an additional slide, which was adjacent to this neuroepithelial region, was available for further analysis. Otx2 expression was observed in all the nuclei of the de-pigmented region with no specific pattern of localisation, suggesting the retention of the RPE phenotype (Fig. 6.8L). Similarly, CRX expression was also observed in all the nuclei of cells within, and outside of the neuroepithelial region, with no particular localised pattern (Fig. 6.8M). Otx2 and CRX expression was observed to be co-localised in all the nuclei of the aggregate (Fig. 6.8K). The positive controls for the immunohistochecmical labelling of both HESC-RPE +/-bFGF, and HESC-RPE +/-bFGF +/- SB431542 are displayed in figure 6.9.

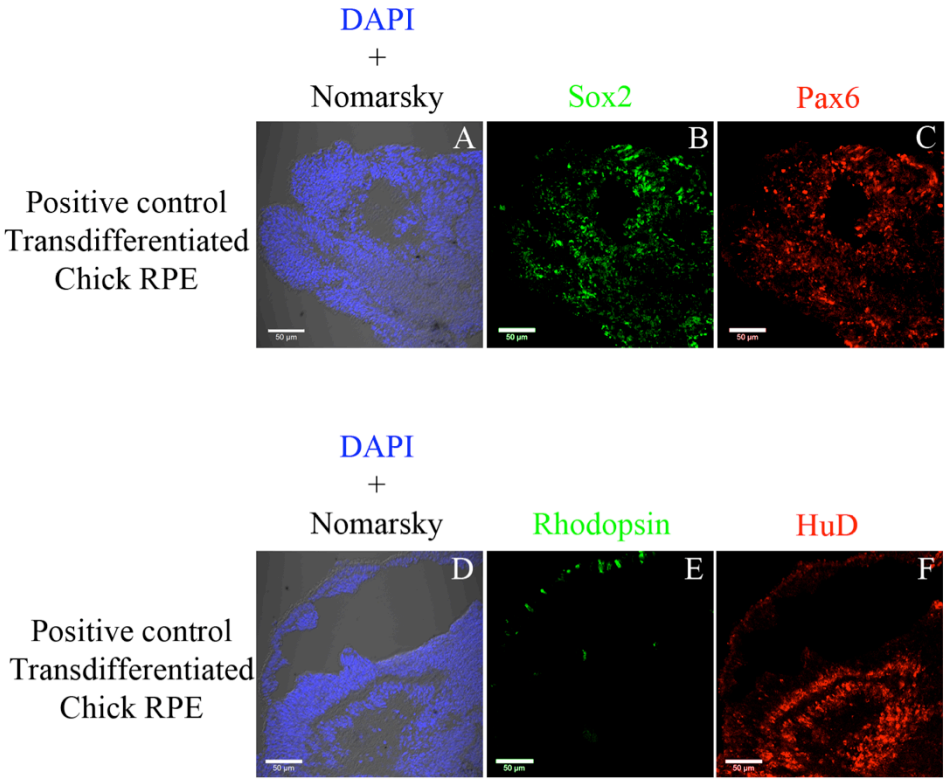


Fig. 6.9
Positive controls for immunohistochemistry for all HESC-RPE experiments. Not all markers were observed in all tissues and so it was necessary to run positive controls for the immuno-labelling for each marker to ensure that the process had been successful. Therefore, labelling of transdifferentiated chick RPE, retinal tissue was performed in parallel to other immunohistochecmistry. Retinal markers Sox2 (B), Pax6 (C), rhodopsin(E), and HuD (F) were all labeled as expected. Scale bars: 50uM.

Once again, image analysis software was employed in order to attempt to quantify the level of pigmentation, and expression of Pmel17 in SB431542+bFGF treated, and DMSO treated HESC-RPE aggregates. SB431542+bFGF treated HESC-RPE did not exhibit a significant difference in the level of pigmentation (n=5) from DMSO treated, negative controls (n=5) (Fig. 6.10A) in a similar manner to that observed for bFGF treated HESC-RPE alone (Fig. 6.6A). The average level of expression of Pmel17 was less in SB431542+bFGF treated HESC-RPE, compared with DMSO treated HESC-RPE (Fig. 6.10B), as expected. However, this difference was not found to be significant. This pattern of Pmel17 expression was similar in profile to bFGF only treated RPE, however, in this instance the difference was not statistically significant (Fig. 6.6). Positive control labelling for all HESC-RPE experiments is displayed in Fig. 6.9.

The fact that a number of HESC-RPE cultures appear to display evidence for the presence of neural retina, it was necessary to analyse the expression profile of these aggregates immediately following their dissection, to ascertain whether or not the presence of retinal markers in cultured explants resulted from the culture conditions themselves, or from contamination of HESC-RPE with neural tissue during the isolation process. HESC-RPE foci dissected from the same super-confluent flasks of human embryonic stem cells as the HESC-RPE foci used in the above experiments, were immediately fixed for immunohistochemical analysis. The majority of foci (n=4) did not appear to exhibit evidence of retinal marker expression or transdifferentiation (Fig. 6.11). These HESC-RPE foci were largely composed of pure, heavily pigmented (Fig. 6.11A), Pmel17 positive (Fig. 6.11D) cells, which did not express neural progenitor marker Sox2 (Fig. 6.11B). However, all HESC-RPE fixed directly from the flask were observed to express Pax6 in the nucleus of Pmel17 positive, pigmented cells (n=5)(Fig. 6.11C, E). Despite the fact that no HESC-RPE foci were observed to express neural marker Sox2, one HESC-RPE aggregate did exhibit the expression of HuD (Fig. 6.12B, D) in the cytoplasm of many non-pigmented cells (Fig. 6.12A) in the aggregate. Pigmented cells in this aggregate did not exhibit the expression of HuD (Fig. 6.12B). No rhodopsin expression was observed in any post-dissection aggregate (Fig. 6.12C).

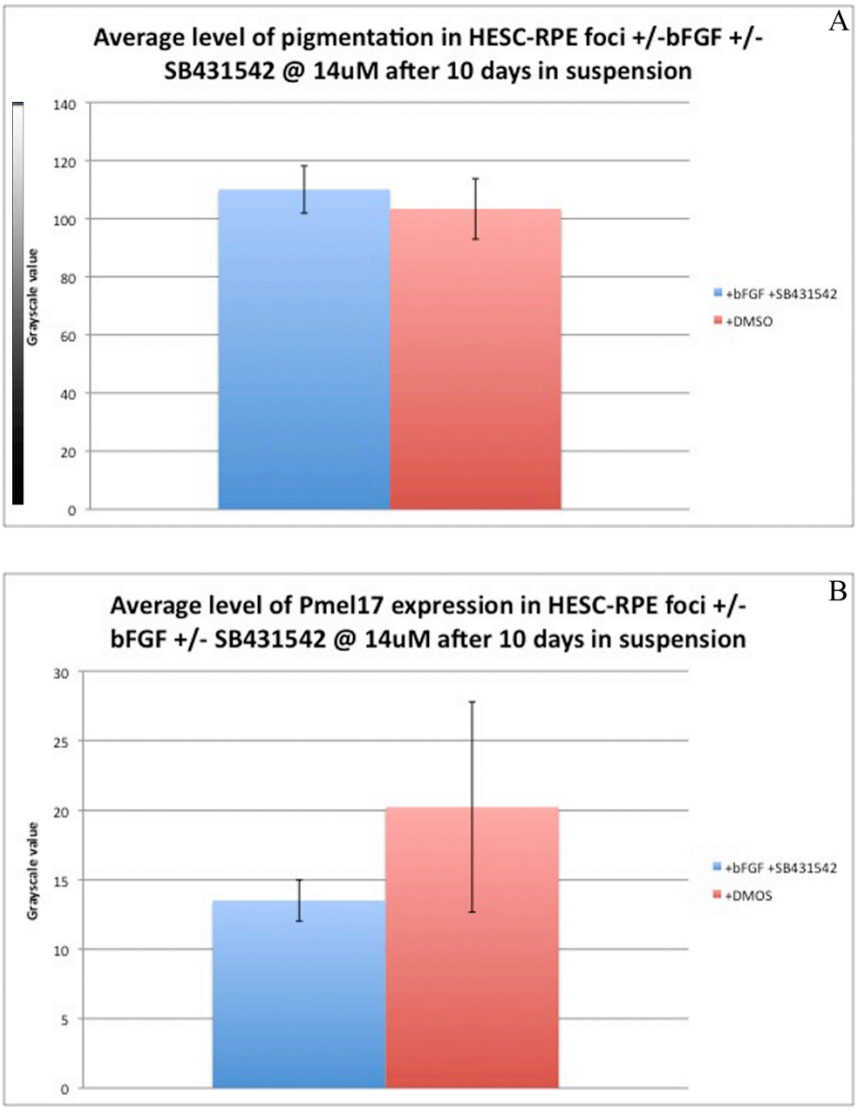


Fig. 6.10
Quantification of the levels of expression of Pmel17 and pigmentation in HESC-RPE non-adherent cultures treated with bFGF+SB431542 or DMSO for 10 days, using image analysis software.
The average expression/pigmentation level was calculated by analysing the average pixel intensity of grayscale images for multiple images in each culture condition. The differences in expression/pigmentation level are therefore expressed on a grayscale (Black = 0, White = 255). (A) There was no significant difference in the level of pigmentation between the two culture treatments. (B) There was no significant difference in the level of expression of Pmel17 between the two culture conditions. Error bars: Standard error.

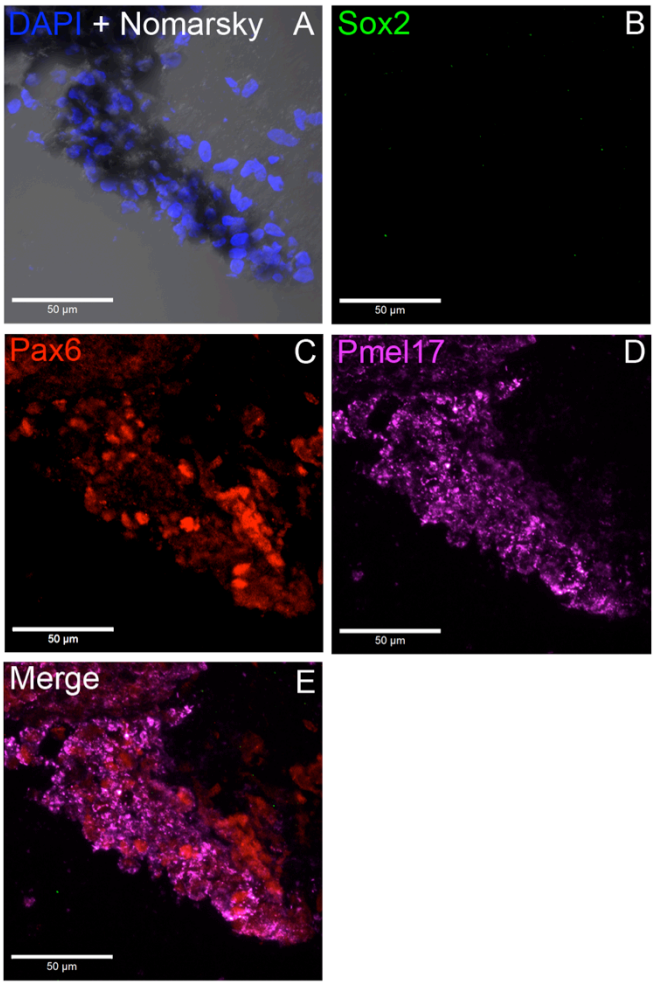


Fig. 6.11
Post-dissection HESC-RPE focus prior to any culture treatment.
A number of HESC-RPE foci were immediately fixed following dissection in order to analyse the initial expression profile of HESC-RPE to act as a control for any changes in gene expression resulting from any culture treatment. All HESC-RPE foci were observed to robustly express Pax6 (C) and Pmel17 (D), in addition to displaying intense pigmentation (A). No Sox2 expression (B) was observed in any aggregate.Scale bars: 50uM.

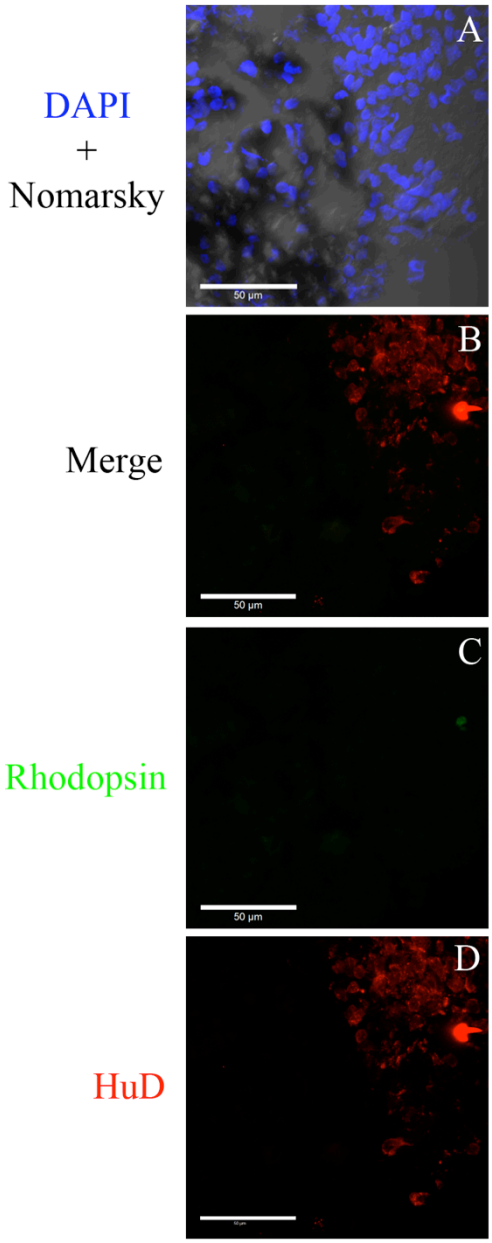


Fig. 6.12
Post-dissection HESC-RPE focus prior to any culture treatment.
A number of HESC-RPE foci were immediately fixed following dissection in order to analyse the initial expression profile of HESC-RPE to act as a control for any changes in gene expression resulting from any culture treatment. No HESC-RPE foci were observed to express rhodopsin (C), however, one aggregate did express the retinal marker, HuD (D) in a largely non-pigmented region of cells (A). This would suggest that some HESC-RPE aggregates contain retinal tissue prior to any further treatment. Scale bars: 50uM.

6.2.4 Discussion:

The majority of HESC-RPE foci, isolated at the earliest possible stage and treated with bFGF, did not exhibit evidence of transdifferentiation. These aggregates retained their pigmented, Pmel17 positive, epithelial phenotype and did not express retinal markers, as would be expected. This may suggest that the HESC-RPE cells had already developed beyond the stage at which they can transdifferentiate in response to bFGF, and therefore retained their RPE phenotype. Interestingly, both bFGF treated and untreated aggregates were negative for Pax6 expression, in contrast with HESC-RPE control foci, which were fixed immediately following dissection. These controls, like expanded HESC-RPE monolayers (Vugler et al., 2008), all exhibited robust Pax6 expression. This begs the question as to why Pax6 expression is down-regulated in HESC-RPE aggregates following 10 days in culture. It is possible that the aggregates are able to continue their maturation in culture, with a subsequent down-regulation of Pax6 reflecting the normal maturation of RPE cells *in vivo*. However, the fact that HESC-RPE monolayers are known to retain robust expression of Pax6 for at least a number of weeks in culture (in the same culture medium), this begs the question as to why non-adherent aggregates down-regulate the expression of the transcription factor, and monolayers do not.

It is possible that some contaminant tissue in the HESC-RPE aggregates is able to mimic the function and signaling of the extraocular mesenchyme in development, which appears to be responsible for the augmentation of the RPE phenotype, as well as a down-regulation of Pax6 (Fuhrmann et al., 2000a, Fuhrmann et al., 2000b). Indeed, transplantation of HESC-RPE cells into the sub-retinal space of the mature rat eye, adjacent to the extraocular mesenchyme at the back of the eye, is sufficient for down-regulation of Pax6 expression in the HESC-RPE (Vugler et al., 2008). However, this seems unlikely given the fact that some aggregates displayed little or no contaminant tissue. In addition, the effects of the growth factor thought to be responsible for the augmentation of the RPE phenotype (via release from the extra-ocular mesenchyme), activin, is known to be antagonized by bFGF

signaling (Sakami et al., 2008, Fuhrmann et al., 2000b, Nguyen and Arnheiter, 2000, Hyer et al., 1998). One might therefore expect a high dose of bFGF treatment to maintain the expression of Pax6 in treated aggregates, which was not observed to be the case. It is possible that the lack of interaction with a substrate somehow allows the HESC-RPE aggregates to release different autocrine signaling pathways, or respond differently to existing ones, in order to regulate the expression of Pax6. The identity of unknown signaling pathways which may be involved in the down-regulation of Pax6 expression are likely to be of paramount importance in elucidating the mechanisms of transdifferentiation, given the apparently heavy involvement of the transcription factor in initiation and maintenance of the phenomenon (Spence et al., 2007b, Azuma et al., 2005a, Kuriyama et al., 2009a).

6.2.4.1 bFGF only:

Despite the majority of HESC-RPE aggregates not exhibiting evidence for transdifferentiation, a couple of aggregates did exhibit some evidence that transdifferentiation may have taken place. One of these aggregates had been treated with bFGF, but the other had not. These HESC-RPE aggregates displayed regions of largely non-pigmented, thickened neuroepithelium which were found to express the expected retinal markers associated with transdifferentiated retina such as Pax6 and Sox2, which co-localised in many cells, which indicates the presence of retinal progenitors. Additionally, these regions were continuous with heavily pigmented, Pmel17 positive, RPE monolayers. The neuroepithelial regions themselves were negative for Pmel17 expression, with the bFGF HESC-RPE aggregate displaying some weak, granular labeling for Pmel17 throughout the thickened region, reminiscent of Pmel17 observed within the native immature retina as previously discussed. This, along with pigment granules present throughout the neuroepithelium of aggregates in both bFGF treated and untreated conditions, would suggest a previous specification as RPE cells. However, it may also imply that these neuro-retinal regions were actually differentiating towards RPE cells, however this seems unlikely. No Pmel17 expression was observed in the

neuroepithelium of the untreated aggregate, which is most likely due to its apparently more developed state, when compared with the similar region in the bFGF treated aggregate. This is evident from its much thicker appearance. It is possible that the neuroepithelial region in bFGF treated aggregates actually results from displaced neural cells, which were attached to the basal side of the HESC-RPE focus. However, this seems unlikely given the continuous, smooth transition between the neuroepithelial region and the pigmented RPE monolayer, including a steady gradient in the change of Pmel17 expression over this region, which implies a shared origin for these RPE and neuro-retinal cells. Unfortunately, owing to the small size of the neuroepithelium, no further immunohistochemical analysis could be performed.

However, the untreated aggregate exhibited a larger region of neuroepithelium, which allowed the expression of differentiated retinal markers, HuD and rhodopsin, to be analysed. No contaminant tissue was present at the basal side of the HESC-RPE monolayer, and the only neuro-retinal tissue present was the neuroepithelial region of interest, which was once again continuous with the pigmented RPE monolayer. This again implies a shared origin of cells for both regions. Perhaps most interestingly, this neuroepithelial region was observed to robustly express HuD, which was primarily localized to the basal surface of the neuroepithelium, which strongly suggests an inverted phenotype, a classical characteristic of transdifferentiated retina (Opas and Dziak, 1994a, Opas and Dziak, 1994b, Sakami et al., 2008). This expression pattern was conserved throughout the length of the neuroepithelium at both ends, which were continuous with pigmented RPE, with no signs of twisting of the tissue. This implies that the inverted phenotype is real, and not as a result of folding or twisting of the tissue. No rhodopsin was observed in this region after 10 days in culture, which is not unexpected given that the human gestation period is much longer than that of the chick, and therefore it is likely that cells would have to be cultured for longer before the retina was mature enough to express this photoreceptor marker.

Quantification of the level of pigmentation and the level of expression of Pmel17 in bFGF treated, and untreated HESC-RPE aggregates revealed a lower level of Pmel17

expression in bFGF treated cells when compared with negative controls, however this difference was not statistically significant. This would suggest that bFGF treated HESC-RPE cells are beginning to lose their RPE phenotype in response to bFGF, however, this difference did not correlate with a significant loss in the level of pigmentation of HESC-RPE cells. This could possibly suggest that the majority of HESC-RPE aggregates are too developmentally mature to respond to bFGF treatment, even when isolated at an early stage post-genesis.

The evidence for HESC-RPE transdifferentiation in both bFGF treated, and untreated aggregates was intriguing because the effect appeared to occur in similar proportions of aggregates (n = 1 for both) independently of exogenous growth factor treatment. This initially implied that the culture system itself may encourage transdifferentiation regardless of exogenous factors, however, the presence of similar evidence of transdifferentiation in post-dissection controls (which also occur in a similar proportion of aggregates) would indicate that these neuro-retinal regions are in fact contaminants in the initial HESC-RPE foci preparation, prior to culture. Intriguingly however, no regions of Sox2 positive cells were observed in any of the post-dissection controls, which may imply that the aggregates develop Sox2 expression as a result of the culture system itself. Despite this, it is entirely possible that Sox2 may be present in some aggregates prior to culture, but was perhaps missed in the batch of controls that were analysed. Despite the implication that neuroepithelial regions appear to be present prior to culture, the fact that the observed neuroepithelia are continuous with pigmented RPE cells, in addition to displaying an inverted phenotype, would suggest that the HESC-RPE has actually undergone transdifferentiation prior the dissection of the foci. This is possible given the likelihood that multi-potent, transdifferentiation competent HESC-RPE cells would come into contact with a wide range of growth factors, including bFGF, during their development in a super-confluent, heterogenous population of spontaneously differentiated HESC's. This lends credence to the idea that human RPE cells do have the potential for transdifferentiation, however, as in animal models (Zhao et al., 1995, Park and Hollenberg, 1989, Park and Hollenberg, 1991, Park and Hollenberg, 1993, Coulombre, 1981, Coulombre and Coulombre, 1965, Pittack et al., 1997, Pittack et al., 1991, Sakami et al.,

2008), this only occurs at the earliest stages of development. Therefore, in order to unlock the transdifferentiation potential of HESC-RPE cells, it will be necessary to attempt to extend the window of competence for transdifferentiation, given the difficulty in obtaining human RPE cells at earlier stages.

6.2.4.2 bFGF + activin inhibitor SB431542:

It has been reported that the window of competence for transdifferentiation of RPE cells in response to bFGF can be extended through inhibition of the activin signaling pathway using a pharmacological inhibitor (Sakami et al., 2008). This is reported to extend the developmental window of competence for transdifferentiation by up to 2 days *in vitro*, which all in likelihood corresponds to approximately 7 days in human development. This may be long enough to aid the transdifferentiation of early generated HESC-RPE foci in response to bFGF.

Unfortunately, the majority of HESC-RPE explants treated with bFGF + SB431542 did not differ from DMSO treated negative controls, in that they retained the characteristically pigmented, Pmel17 positive, RPE phenotype. No retinal markers were observed in these cultured aggregates and once again, Pax6 was found to have been down-regulated in all of the aggregates, treated and untreated. This would tend to suggest that the activin signaling pathway is not involved in the abolition of Pax6 expression within the HESC-RPE cells after 10 days, given its reported involvement in both the establishment and maintenance of the RPE phenotype (Fuhrmann et al., 2000b), and its reported expression in both extraocular mesenchyme (which may contaminate the aggregates), and the RPE themselves (Sakami et al., 2008). If activin signaling was responsible for down-regulation of the transcription factor then Pax6 expression would be expected to be maintained in bFGF + SB431542 treated aggregates, which it was not. However, one bFGF + SB431542 treated HESC-RPE aggregate did appear to show some signs of potential transdifferentiation.

In this instance, a region of somewhat de-pigmented, thickened tissue was continuous with a more heavily pigmented RPE monolayer, and was observed to express both Pax6 and Sox2, suggesting the presence of retinal progenitor cells. This region was not completely neuroepithelial in appearance when compared with other neuroepithelial regions discussed previously, owing to the fact that a significant amount of pigment was often still present in some sections. Additionally, the expression of HuD was scattered throughout the region, rather than localizing to a particular side of the region as in native retina. The region exhibited a phenotype that was half reminiscent of a presumptive retina, and half retained RPE characteristics, including robust Pmel17 expression. The presence of Otx2 in all cells in this region would support the fact that the RPE-like phenotype was maintained, however, CRX expression was found to co-localise with Otx2 in all cells of the aggregate, as it was in native, human fetal eye tissue discussed previously. Despite the fact that Crx is normally associated with photoreceptor development (Rutherford et al., 2004, Jomary and Jones, 2008, Peng and Chen, 2007, Hennig et al., 2008, Freund et al., 1997) (Glubrecht et al., 2009, Freund et al., 1997, Furukawa et al., 1997), it is also reportedly expressed in RPE cells (Esumi et al., 2009). It is possible that the antibodies used to label both of these related transcription factors may have cross-reacted and labeled similar epitopes on both proteins.

No significant differences were observed in the levels of pigmentation or Pmel17 expression between bFGF + SB431542 treated and DMSO treated HESC-RPE aggregates. In fact, a pattern similar to that of HESC-RPE treated +/-bFGF only was observed, with an apparent decrease in Pmel17 expression for bFGF + SB431542 aggregates, however, in this instance the error bars over-lapped, indicating that this difference was not significant. It is likely that the difference observed for +/-bFGF treated HESC-RPE was not significant given the correlation between the patterns of expression for both experiments.

Despite the fact that no transdifferentiated structures were observed in negative control cultures, it is likely that once again, the neural tissue observed in bFGF + SB431542 treated cultures was not as a result of the culture treatment, but as a result of some kind of differentiation which occurred prior to the beginning of culture. This appears to be the case

because the number of aggregates exhibiting the presence of neural-like tissue in this experiment did not exceed the same proportion of aggregates observed to contain neural tissue in the post-dissection controls, despite the fact that no Sox2 expression was observed in these controls. This would therefore suggest that the inhibition of activin signaling was not sufficient to increase the likelihood of HESC-RPE to transdifferentiate in response to bFGF. It may be that the RPE cells had already developed beyond the point of development at which activin inhibition is thought to be no longer effective, as observed in the embryonic chick model (Sakami et al., 2008). In which case, this once again begs the question as to what restrictive mechanism is affecting the ability of later stage RPE cells to undergo transdifferentiation in response to bFGF.

6.2.4.3 General discussion:

Future experiments will focus on elucidating the mechanisms behind this loss in the potential for transdifferentiation. However, it is possible that a different dose of activin inhibitor will be required in order to elucidate an effect, despite the fact that the concentration of SB431542 employed in this experiment was sufficient to be effective in the chicken model of transdifferentiation (Sakami et al., 2008). Given the apparent similarities between the two species in this regard, this seems unlikely. The likelihood is that it will also be necessary to culture the aggregates for longer once transdifferentiation is initiated, in order to give time for the photoreceptors the develop. This may also require further optimization of the culture process in order to support the growth and development of the resulting retinas.

Chapter 7 –

General discussion & conclusions

This investigation did suggest that human RPE cells are able to undergo a process of limited transdifferentiation towards a rudimentary, neuroepithelial phenotype, at the earliest stages of ocular development. To my knowledge, this is the first demonstration of the potential for human RPE cells to undergo classical transdifferentiation. This process appears to be equivalent to that of classical animal models of the phenomenon, including the embryonic chick. The evidence for this is both direct and indirect.

The direct evidence for human RPE transdifferentiation comes in the form of a phenotypic change in early stage, cultured, human fetal RPE explants, in response to treatment with exogenous bFGF in a non-adherent culture system. This was shown to yield small regions of apparently transdifferentiated neuroepithelium, which expressed markers of retinal progenitors after 10 days in culture. Despite this, most of the cells in these RPE explants retain the characteristic RPE pigmentation and expression of Pmel17, suggesting that transdifferentiation has not taken place in these regions. This would indicate that the development of the RPE is heterogenous across the RPE monolayer, with some regions retaining the capacity to transdifferentiate in response to bFGF, while in contrast, others appear to lose this potential with on-going development.

The indirect evidence for classical human RPE transdifferentiation are the presence of thickened, neuroepithelial structures in isolated HESC-RPE foci, which are largely non-pigmented, express markers of retinal progenitors, in addition to differentiated retinal ganglion cells. These structures are continuous with pigmented monolayers regions, which retain the RPE phenotype, including the expression of Pmel17. This investigation revealed evidence to suggest that these structures did not result from their treatment in culture, but are likely to have arisen during genesis in super-confluent flasks on HESC's, because retinal structures were also found in post-dissection controls. One of the thicker, more developed neuroepithelial structures observed in HESC foci exhibited the localized expression of ganglion cell marker, HuD, in a single-layered, clearly organized, fashion, reminiscent of a ganglion cell layer in native retina. This again suggests that this structure was indeed a developing neural retina. However, the fact that this HuD expression was localized to the

basal layer of the neuroepithelium suggested an inverted phenotype, characteristic of transdifferentiated retina. This indicates that the source of this retinal tissue was from transdifferentiation, and implies that the RPE monolayer had undergone transdifferentiation towards a neural retina phenotype prior to isolation and culture. It would be interesting to examine whether or not human fetal RPE at even earlier stages of development than those investigated in this study are able to exhibit more robust transdifferentiation in response to bFGF, as is the case for animal models of the phenomenon. Should this tissue become available, the approximate equivalent stage to chick HH24 in human would likely be CS16 in human RPE cells, immediately following pigmentation.

Transdifferentiation of embryonic chick RPE in response to exogenous bFGF treatment, an established model of the phenomenon, was also shown to occur in a humanized system with human medium and growth factors. The capacity for transdifferentiation was not enhanced or inhibited by the use of undefined, human components, and gave similar results as previously reported media. This included the full transdifferentiation of embryonic chicken RPE explants (stage HH24/E4) to produce a non-pigmented, retinal neuroepithelium, which exhibited the expression of a number of developing retinal markers including Pax6 and Sox2, in addition to differentiated markers of specific retinal cell types, including Islet-1 (ganglion cell), HuD (ganglion/amacrine cell) and rhodopsin (rod photoreceptors). This would suggest that the development of the transdifferentiated retina largely resembles that of the native retina, which implies that transdifferentiated retina could potentially be a good model for the study of early retinal development, as well as potentially for the production of useful, retinal cells for on-going development of transplantation strategies for various retinal diseases. It may be useful to further test the capacity of RPE transdifferentiation to provide functional retinal neurons by establishing an *in vitro* assay which is able to measure the capacity of these cells to respond to light.

The capacity for embryonic chick RPE transdifferentiation in response to bFGF *in vitro* was not observed to be lost at a stark point of development at HH25 as had been previously reported (Sakami et al., 2008, Pittack et al., 1997, Pittack et al., 1991). Instead, the

capacity for transdifferentiation was exhibited until at least HH27 in a variable manner, with some explants exhibiting regions with more robust transdifferentiation than others, which displayed very few features of transdifferentiation, if any. It appears as though the ability to transdifferentiate declines more gradually after the reported HH24, which was still the gold standard for full transdifferentiation. The decline in transdifferentiation potential did not appear to be dependent on the HESC medium used, as similar results were observed in a control medium, previously reported to facilitate transdifferentiation up until HH25/E5. It is unsurprising that a developmental loss in the potential for transdifferentiation is observed to be more gradual than previously reported, given the apparent heterogenous development of the RPE monolayer. The potential for transdifferentiation appeared to be regionally restricted, which would also support this idea, however, it is unclear what factors cause this to be the case. A similar variability in the level of expression of Pax6, an important transcription factor in transdifferentiation (Bharti et al., 2012, Spence et al., 2007b, Azuma et al., 2005a), was observed to occur after stage HH24 in chick RPE, which may implicate a down-regulation in Pax6 as a crucial step in the restriction of the capacity for transdifferentiation after this stage.

Despite the apparent correlation between the gradual, regional down-regulation of Pax6, and the gradual decline in the capacity for transdifferentiation, it may not necessarily be the only critical factor involved in conferring the capacity for RPE transdifferentiation, given the fact that over-expression in chick RPE alone at later stages is not able to induce transdifferentiation in all RPE cells (Azuma et al., 2005a). Additionally, Pax6 expression is maintained in some human fetal RPE explants after 10 days in culture, as well as HESC-RPE cultures which robustly express the transcription factor, both of which did not necessarily exhibit robust signs of the onset of transdifferentiation in response to bFGF. This would imply that other key factors are necessary in conjunction with Pax6 for the initiation of transdifferentiation. It may also suggest that a threshold level of Pax6 expression is required before transdifferentiation may take place, which is has not been met in these RPE cell cultures. The likely-hood is that both of these factors will be applicable, given the loss in potential for ectopic Pax6 alone to induce transdifferentiation at later stages, in addition to

RPE cells expressing Pax6 as a normal feature of development without undertaking a phenotypic change. Similarly, the action of Pax6 has been reported to operate in a complex feedback loop in order to both augment the RPE phenotype (Bharti et al., 2012, Baumer et al., 2003), as well as initiate a departure from it (Bharti et al., 2012, Azuma et al., 2005a, Spence et al., 2007b), which may imply the presence of a regulatory switch that changes the transcriptional output of Pax6. If this is the case, manipulation of such a molecular switch is likely to be important if the capacity for transdifferentiation is to be developmentally extended.

One of the more interesting results to come from this investigation is the observed changes in the expression of Pax6 when RPE cells are removed from their normal environment. Particularly, HESC-RPE, which under normal, adherent, culture conditions, retains the robust expression of Pax6 over time in culture. However, when cultured in the standard, non-adherent, transdifferentiation culture system, the majority of these cells lose the expression of Pax6. The reason for this is unclear, however, given the important nature of Pax6 in the transdifferentiation of RPE cells, it could be likely that any candidate responsible for this down-regulation might also be responsible for the inhibition of transdifferentiation.

Transdifferentiation of the RPE appeared to reflect the multi-potent behavior of the progenitor cells present in the developing optic cup, with neural retina specifying factors such as FGF's supporting the progression of the phenomenon, and RPE specifying factors inhibiting the phenomenon (Sakami et al., 2008, Muller et al., 2007, Zhang and Yang, 2001, Spence et al., 2004, Bharti et al., 2012, Fujimura et al., 2009). Inhibition of the RPE augmentation factor activin had been previously reported to increase the capacity for transdifferentiation of embryonic chick RPE in response to bFGF, and ectopic activin treatment had been reported to antoganise the effects of bFGF, thereby inhibiting transdifferentiation (Sakami et al., 2008). Despite this, activin inhibition was only reported to partly increase the capacity for transdifferentitation of RPE cells, which consequently implied the action of other pathways in restriction of transdifferentiation. Therefore, two pathways, BMP and Shh, which had both been implicated in RPE augmentation, were tested for their

capacity to inhibit transdifferentiation. BMP signaling factors were found not to modulate Pax6 expression in cultured RPE explants, and did not inhibit bFGF-mediated transdifferentiation. This would suggest that BMP signaling is able to augment the RPE fate in an indirect manner, via regulation of secondary signaling pathways *in vivo*. Similarly, exogenous Shh was not able to inhibit bFGF-mediated transdifferentiation, once again suggesting an indirect action for this signaling pathway in augmentation of the RPE fate *in vivo*, possibly via modulation of the expression of FGF's in the retina (see chapter 4 for discussion). However, another study reported that Shh still elicited inhibition of the FGF signaling pathway in retinectomized eyes, implying a possible direct action in the RPE. It is therefore unclear why exogenous Shh is not able to inhibit bFGF-mediated transdifferentiation *in vitro*. It is possible that other, non-retinal derived signaling events are necessary for inhibition of FGF in retinectomized eyes, or it may be that Shh is only inhibitory at certain doses. This pathway will require further investigation in order to elucidate its precise role in the reported inhibition of transdifferentiation.

Activin inhibition did not enhance the capacity for bFGF-mediated transdifferentiation of HESC-RPE cells at all, when compared with bFGF treatment alone. It is unclear why this is the case and may be that human RPE cells behave differently from their chick counterparts, however, this seems unlikely given the apparent conservation of the phenomenon across a number of species. More likely is that HESC-RPE have already developed beyond the point at which activin inhibition is effective in enhancing transdifferentiation, or that activin signaling is no longer the limiting factor for restriction of transdifferentiation, and other pathways are instead responsible. If this is the case then the identity of these pathways are unknown.

Future experiments will focus on elucidating the identity of those factors responsible for limiting the capacity for bFGF-mediated transdifferentiation in RPE cells. If these restrictions can be alleviated, it may be possible to restore the capacity for transdifferentiation in some, if not all RPE cells. These studies would need to encompass the investigation of known RPE augmentation pathways, including the aforementioned Shh, and Wnt/β-catenin

signaling, which result in transdifferentiation if inhibited. Additionally, if these pathways do not prove to be key in restricting transdifferentiation, it may be necessary to employ broader techniques, such as comparative gene fishing, and high-throughput sequencing arrays (for example Ion torrent), in order to identify new the best candidates for further consideration. These studies would centre around the comparison of the transcription profiles of embryonic chick RPE cells pre- and post- HH24 at a number of stages in order to attempt to identify changes that correlate with a loss in the potential for transdifferentiation. Similarly, it may be useful to use these techniques to compare the very early changes in the transcriptional profile of explanted RPE cells +/-bFGF, in order to define those factors which are crucial for the initial onset, as well as the progression of transdifferentiation.

This investigation has shown that human RPE transdifferentiation may provide a useful tool in understanding early retinal development if human RPE cells can be reliably transdifferentiated in culture. The phenomenon may also provide a useful source of retinal cells for further study of disease, as well as for potential transplantation therapies for disorders effecting the neural retina. If transdifferentiation is to be an effective technique for application in this manner, it will be crucial to elucidate the molecular mechanisms that govern the initiation and progression of transdifferentiation, in order to enhance transdifferentiation of the available sources of human RPE cells, such as HESC-RPE.

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